REMARKS/ARGUMENTS

Specification

The substituted paragraph on page 1 was amended merely to correct the information relating to government funding for research.

Claims

Claims 3-24, 33 are currently pending. Claim 3 has been canceled. Claims 1,2, 25-32, and 34-76 have been withdrawn as directed to a non-elected invention. Claims 77-86 have been added. Claims 4-6, 8-11, 19, and 22 have been amended.

Claims 4, 8, 11, 19, 22, and 33 were amended merely to correct their dependencies. Claims 5 and 6 were amended to recite an isolated polynucleotide "having ABC1 activity". Claims 8-10 were amended to insert the word "pharmaceutically" before the term "suitable carrier", as requested in the Office Action. Support for the claim amendments for 5,6, and 8-10 can be found throughout the specification.

New claims 77-80 are directed to the subject matter of original claim 3, which has been canceled. New claims 81-83 are directed to the subject matter of original claim 11, which originally depended from claim 3 and has been amended in this response. New claims 84-86 are directed to the subject matter of claim 14, which depended from original claim 11. Support for all of these amendments can be found in the original claims as explained above and throughout the specification.

No new matter has been added by way of these amendments.

Claim Objections

Claims 8-10 were amended to insert the word "pharmaceutically" before the term "suitable carrier" to address the objections cited in the Office Action.

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Discussion of the 35 U.S.C. § 112 Rejections

Enablement

Claims 3-24 and 33 were rejected under 35 USC § 112, first paragraph, as allegedly not enabled. Applicants respectfully traverse the rejection.

The Office alleges that while the specification is enabling for a polynucleptide comprising SEQ ID NO: 1 (new claim 77); a polynucleotide encoding a polypeptide comprising SEQ ID NO: 2 (new claim 78); and a polynucleotide comprising nucleotides 291-7074 of SEQ ID NO: 1 (new claim 79), it does not reasonably provide enablement for any polynucleotide comprising a polynucleotide that encodes a polypeptide that is 98% identical to SEQ ID NO: 2 (new claim 80) or any polynucleotide comprising a nucleotide sequence that has either 90% or 95% sequence identity with SEQ ID NO: 1 (claims 5 and 6).

Under 35 U. S. C. § 112, all that is required is that the specification describe the invention in such terms as to enable a person skilled in the art to make and use the invention. Thus, with respect to claim 80, the specification must teach one skilled in the art how to make and use a polynucleotide having ABC1 activity encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2. With respect to claims 5 and 6, the specification must teach one skilled in the art how to make and use a polynucleotide having ABC1 activity comprising a nucleotide sequence that has either 90% or 95% sequence identity with SEQ ID NO: 1. The test for enablement is whether one reasonably skilled in the art (1) could make and use the invention (2) from the disclosures in the patent coupled with information known in the art (3) without undue experimentation. *In re Wands*, 858 F.2d 731 (Fed. Cir. 1988); *United States v. Telectronics, Inc.*, 857 F.2d 778 (Fed. Cir. 1988); M.P.E.P. § 2164.01.

Contrary to the Office's allegation, the instant specification provides considerable guidance to enable a skilled artisan to make and use a polynucleotide having ABC1 activity encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2. For example, the specification describes polypetides having at least 98% sequence identity with SEQ ID NO: 2 at, for example, pages 22, 25, and 31. In addition, the specification describes polypeptide variants and defines the biological activity of a

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polypeptide variant at pages 22 and 25. The specification further provides several examples of the types of polypeptide variants and teaches how to design polypeptide variants at pages 23, and 25-28, including teaching the deletion or substitution of non-conserved amino acids as well as conservative amino acid substitution (pages 23 and 27), among others. The specification teaches several methods for identifying and making suitable ABC1 variants at pages 26-28, including structure-function studies and genetic engineering techniques. The specification also teaches that the ABC1 variants can be naturally-occurring or artificially constructed at pages 27 and 28.

Likewise, the instant specification provides considerable guidance to enable a skilled artisan to make and use a polynucleotide having ABC1 activity comprising a nucleotide sequence that has either 90% or 95% sequence identity with SEQ ID NO: 1. For example, the specification describes polynucleotides having at least 90% or 95% sequence identity with SEQ ID NO: 1 at pages 22 and 31. In addition, the specification describes polynucleotide variants and provides examples of polynucleotide variants having biological activity, including those having additions, deletions, and silent substitutions, such as those due to degeneracy of the code at, for example, pages 23, 27, 31 and 39. The instant specification also teaches that the polynucleotides can be produced chemically, enzymatically, or metabolically (specification at pages 19 and 27) and provides a detailed description of the recombinant methods and techniques used to produce the polynucleotides of the invention, including making and amplifying the polynucleotides (specification at, for example, pages 35-40, 43-44, and the Examples). The specification further teaches the sequencing of the polynucleotides and how to determine the percent identity with SEQ ID NO: 1 or a fragment thereof (specification at, for example, 20-22 and 76-79, Example 4). While the recombinant techniques described throughout the specification and in the Examples (particularly Example 4) were used to produce *exemplary polynucleotides* corresponding to SEQ ID NO: 1, these same techniques and other standard recombinant techniques widely known in the art and easily within the skill of the ordinary artisan can be used to produce additional polynucleotides having at least 90% or 95% identity to SEQ ID NO: 1.

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Further, the specification provides a detailed description of the methods used to test the claimed polynucleotides for ABC1 activity, including the construction of expression vectors, transfection into host cells and assays for determining cholesterol efflux and ABC1 activity (specification at pages 44-51, 71-74, 79-81 and Examples 1, 6-8).

The specification also teaches one skilled in the art how to use the claimed polynucleotide molecules at, for example, pages 44-61, 67-68, and the Examples section.

The Office alleges that the scope of the claims are not commensurate with the enablement provided by the disclosure because the claims encompass an enormous number of polynucleotide sequences. Further, the Office alleges that while recombinant and mutagenesis techniques are known, it is not routine to screen for substitutions or modifications of nucleotides and given that the results of such changes are unpredictable, the reasonable expectation of success in obtaining the desired activity is limited. Finally, the Office alleges that "changing the nucleotides sequences as proposed by the applicants... may not lead to desired function of the polynucleotides." (Office Action at page 4).

First, given that the present claims are limited to those polynucleotide sequences that have ABC1 activity, they do not encompass an infinite number of polynucleotides as the Office suggests. Second, the specification clearly teaches nucleotide and amino acid substitutions, additions, and deletions that *maintain* the ABC1 activity and teaches to avoid changing nucleotide and/or amino acids that significantly alter the activity of the ABC1 protein. Given that the claims are limited to those polynucleotides encoding a polypeptide having ABC1 activity, variant polynucleotides that do not have ABC1 activity are not within the scope of the present claims.

In addition, as discussed above, the specification teaches one skilled in the art how to produce the polynucleotides of the invention using well-established and standard recombinant techniques that are well within the knowledge and skill of the ordinary artisan. Applicants submit that it is routine experimentation to use recombinant, mutagenesis, amplification, and sequencing techniques to make and determine the sequences of variant polynucleotides. Applicants further submit that it is a matter of routine experimentation to screen variant nucleotide sequences for ABC1 activity using

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the techniques described in the specification and other well-known techniques. The law clearly states that "a considerable amount of experimentation is permissible, if it is merely routine." *In re Wands,* 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Further, the fact that experimentation may be complex does not necessarily make it undue. *Massachusetts Institute of Technology v. A.B. Fortia,* 774 F.2d 1104 (Fed. Cir. 1985); *In re Wands,* 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Thus, the test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. *In re Angstadt,* 537 F.2d 498 (CCPA 1976). Applicants submit that such routine work as producing variant polynucleotides and screening them to determine activity is well within the knowledge and skill of the ordinary artisan and does not involve undue experimentation.

The Office further alleges that the specification does not support the broad scope of the claims because the specification does not establish regions of the polynucleotide sequence that can be modified without affecting activity, the general tolerance of the ABC1 sequence to modification, and a rational and predictable scheme for modifying the sequence with the expectation of obtaining the desired activity. However, with respect to claim 80 (drawn to a polynucleotide encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2), the specification clearly defines and teaches methods for the determination of percent sequence identity at pages 23-28, which methods are routine in the art and within the skill of the ordinary artisan. Further, the specification establishes regions of the polynucleotide sequence that can not be modified without affecting activity by teaching the structural/functional domains relating to ABC1 transporter activity, as well as teaching highly conserved sequences (specification at page 17). The specification additionally teaches examples of specific amino acid substitutions that are not tolerated at pages 28-29 (see SEQ ID NOs: 8 and 10) and 72-74 (Example 1 and corresponding Figure 1) and several amino acid substitutions that are tolerated at page 30.

In addition to the teachings in the specification, the structural/functional domains correlating to its activity as a transporter protein, as well as the regions conserved between different ABC transporter proteins and across species, were characterized and

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known in the art at the time of filing the present application. See, for example, Broccado et al, *Biochim. Biophys. Acta*, 1461: 395-404 (1999) and Hayden et al, *Curr. Opin. Lipid.*, 11: 117-122 (2000), both of which are submitted herewith as Exhibit 1. Among other things, Broccado et al. describes sequences conserved between different ABC transporter proteins and across species. Broccado et al., also describes the structural domains in the ABC1 protein and the corresponding polynucleotide domains, as well as provides examples of amino acid residues known to be conserved and others known to be variable. Likewise, Hayden et al, describes the structural domains of ABC1, as well as critical residues that do not tolerate substitution and residues that tolerate substitution. Based on the teachings in the specification, as well as structural information known in the art, a skilled artisan would realize that the nucleotide sequence corresponding to the characterized structural/functional domains would tolerate only limited modification. Further, one skilled in the art would realize that the highly conserved sequences would tolerate limited modification, if any. Further, a skilled artisan would have realized which specific residues could be substituted and which residues could not tolerate substitution.

With respect to claims 5 and 6 (drawn to polynucleotides having 90% and 95% sequence identity with SEQ ID NO: 1, respectively), as discussed above, the specification establishes regions of the polynucleotide sequence that can not be modified without affecting activity by teaching the structural/functional domains relating to ABC1 transporter activity, as well as teaching highly conserved sequences (specification at page 17). In addition, the specification teaches examples of specific nucleotide substitutions that are not tolerated at pages 28-29 (see SEQ ID NOs: 8 and 10) and 72-74 (Example 1 and corresponding Figure 1) and several nucleotide substitutions that are tolerated at page 30. The specification also establishes regions of the polynucleotide sequence that cannot be modified without affecting activity by teaching the sequence and position of transcription regulatory elements in the 5' untranslated region (specification at pages 32-33, Figure 13) as well as sites in the 3' untranslated region (specification at page 33). One skilled in the art would realize that these sites, as well as other known and published sites in the 5' and 3' untranslated regions, would tolerate limited modification, if any. In

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addition, the ABC1 activity of sequence variants can easily be tested using the methods described in the specification as well as other routine methods known in the art.

For the reasons discussed above, the claims are fully enabled by the specification. Accordingly, the Applicants respectfully requests withdrawal of the 35 U.S.C. § 112 rejections.

Written Description

Claims 3-24 and 33 were rejected under 35 USC § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor(s) had possession of the claimed invention at the time the application was filed. Applicants respectfully traverse the rejection.

To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art could reasonably conclude that the inventor had possession of the claimed invention. *See, e.g., id.* at 1116; M.P.E.P § 2163(I). There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed. M.P.E.P. § 2163(I)(A) (citing *In re Wertheim*, 541 F.2d 257, 263, 191 U.S.P.Q. 90, 97 (C.C.P.A. 1976)). Thus, a description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. *See, e.g., In re Marzocchi*, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971); M.P.E.P. § 2163.04. Therefore, the Office must have a reasonable basis to challenge the adequacy of the written description and has the initial burden of presenting, by a preponderance of the evidence, why a person skilled in the art would not recognize in an Applicant's disclosure a description of the invention defined by the claims. *See, e.g., In re Wertheim*, 191 U.S.P.Q. 90, 97 (C.C.P.A. 1976); *Ex parte Sorenson*, 3 USPQ2d 1462 (Bd. Pat. App. & Int. 1987); M.P.E.P. § 2163.04.

Whether the specification shows that an applicant was in possession of the claimed invention is a factual determination. M.P.E.P. § 2163(I). Possession is shown "by describing the claimed invention with all of its limitations using such descriptive

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means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention." M.P.E.P. § 2163.02 (citing *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572 (Fed. Cir. 1997)). Factors to be considered in determining whether there is sufficient evidence of possession include: (1) the level of skill and knowledge in the art; (2) partial structure; (3) physical and/or chemical properties; (4) functional characteristics alone or coupled with a known or disclosed correlation between structure and function; (5) and the method of making the claimed invention. *Id.* at (II)(A)(2)-(3)(a). Disclosure of *any* combination of such identifying characteristics that distinguish the claimed invention such that one skilled in the art would conclude that the applicant was in possession of the claimed species is sufficient to satisfy written description. *Id*; *see Reagents of the Univ. of Calif. v. Eli Lilly*, 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1406. Correspondingly, as the Patent Office's internal guidelines assert, "the written description requirement may be satisfied through disclosure of function and minimal structure when there is a well-established correlation between structure and function." M.P.E.P. § 2163(II)(A)(3)(a)(i)(C)(2). Thus, when "knowledge and level of skill in the art is high, a written description question *should not be raised* for original claims even if the specification discloses only a method of making the invention and the function of the invention." *Id.* (emphasis added), *see In re Hayes Microcomputer Products, Inc. Patent Litigation*, 982 F.2d 1527, 1534-35, 25 U.S.P.Q.2d 1241, 1246 (Fed. Cir. 1992) ("An applicant's disclosure obligation varies according to the art to which the invention pertains.").

Moreover, that which is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. M.P.E.P. § 2163 (citing *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986)). Further, the written description requirement does not require an actual reduction to practice. M.P.E.P. § 2163. Accordingly, an Applicants need not show that the invention will work for its intended purpose to satisfy the written description requirement.

The Office alleges that the specification does not contain any disclosure of the function of all DNA sequences that are 90% or 95% identical to SEQ ID NO: 1 or the function of polypeptide that is 98% identical to SEQ ID NO: 2. Applicants have

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amended the claims to recite that the variant polynucleotide sequences have ABC1 activity. Accordingly, the function of all of the presently claimed polynucleotides is ABC1 transporter activity.

In making its rejection, the Office seems to allege that an actual reduction to practice is required to meet the written description requirement. However, as discussed above, an actual reduction to practice is not the standard for written requirement. Instead, the specification need only describe a combination of identifying characteristics, such as partial structure, physical and/or chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention.

In this regard, the specification thoroughly describes the partial structure of the claimed polynucleotides. For example, the specification clearly provides the structure (sequence) of SEQ ID NO:1 in providing the 10,442 bp nucleotide sequence of SEQ ID NO: 1 and the structure (sequence) of SEQ ID NO:1 in providing the 2261 amino acid sequence of SEQ ID NO:2. The specification also clearly describes and provides several examples of variant polynucleotide sequences of SEQ IF NO: 1 and SEQ ID NO: 2, as discussed in detail above (specification at, for example, pages 22-28, 31, and 39).

In addition, the specification clearly teaches the ABC1 transporter function of the claimed polynucleotides, provides demonstration of ABC1 transporter activity in Examples 1, 6-8, and teaches sequences to maintain ABC1 transporter activity at, for example, pages 26-30, 32-33, and 72-74, Example 1 and corresponding Figure 1, Figure 13.

In addition to teaching the structure and function of the claimed polynucleotides, the specification also contains a thorough description of how to make and test the claimed variant polynucleotides for ABC1 activity. For example, the specification teaches how to make the claimed polynucleotides throughout the specification and particularly at pages 26-28 (structure-function studies and genetic engineering techniques) and pages 35-40, 43-44, and the Examples (recombinant methods and techniques). The specification further teaches how to test the claimed polynucleotides for ABC1 activity at pages 44-51, 71-74, 79-81 and Examples 1, 6-8. Using these techniques and other techniques well-

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known in the art, one skilled in the art could easily make and test the ABC1 activity of variant polynucleotides having 90% or 95% identity to SEQ ID NO: 1 or variant polynucleotides encoding a polypeptide having 98% identity to SEQ ID NO: 2.

Finally, the specification teaches a correlation between ABC1 sequence and activity by teaching the structural/functional domains relating to ABC1 transporter activity, as well as teaching highly conserved sequences (specification at page 17). The specification also teaches examples of specific amino acid substitutions that are not tolerated at pages 28-29 (see SEQ ID NOs: 8 and 10) and 72-74 (Example 1 and corresponding Figure 1) and several amino acid substitutions that are tolerated at page 30. The specification also teaches a correlation between ABC1 sequence and activity by teaching the sequence and position of transcription regulatory elements in the 5' untranslated region (specification at pages 32-33, Figure 13) as well as regulatory sites in the 3' untranslated region (specification at page 33).

Based on the teachings in the specification of the structure of the claimed polynucleotides, its functional characteristics, the correlation between its structure and function, and the methods of making the claimed polynucleotide molecules, as outlined above, Applicants have clearly described identifying characteristics that distinguish the claimed polynucleotide molecules, such that one skilled in the art would conclude that Applicants were in possession of the claimed invention.

For the reasons discussed above, the claims satisfy the written description requirement. Accordingly, the Applicants respectfully requests withdrawal of the 35 U.S.C. § 112 rejections.

Discussion of the 35 U.S.C. § 102/103 Rejections

35 U.S.C. § 102(b)/103(a) Rejection in view of Langmann et al.

The Office rejected claims 3-24 and 33 under 35 U.S.C. § 102(b) as allegedly being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as allegedly obvious over Langmann et al (Swiss Protein Accession No. O95477; GenBank Accession No. AJ012376 and BBRC, April 1999, 257(1):29-33). The rejection is respectfully traversed.

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New claims 77-80 correspond to the subject matter of original claim 3. Claim 77 is directed to an isolated polynucleotide comprising SEQ ID NO: 1. Claim 78 is directed to an isolated polynucleotide encoding a polypeptide comprising SEQ ID NO: 2. Claim 79 is directed to an isolated polynucleotide comprising nucleotides 291-7074 of SEQ ID NO: 1. Claim 80 is directed to an isolated polynucleotide encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2.

Under 35 U.S.C. § 102(b), a claim is anticipated only if each and every element as set forth in the claim is found in a single art reference. *Verdegaal Bros. v. Union Oil Co.*, 814 F.2d 628, 631, 2 USPQ2d 1051, 10533 (Fed. Cir. 1987); *In re Recombinant DNA Technology Patent and Contract Litigation*, 30 USPQ2d 1881 (S.D. Ind.1993) ("A patent is anticipated only if all the elements and limitations of the claims are found within a single, prior art reference."); *Structural Rubber Products Co. v. Park Rubber Co.*, 749 F.2d 707, 716 (Fed. Cir. 1984) (All elements of the claimed invention must be contained in a single prior art disclosure and must be arranged in the prior art disclosure as in the claimed invention); M.P.E.P § 2131. Furthermore, no difference may exist between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of invention. *In re Recombinant DNA Technology Patent and Contract Litigation*, 30 USPQ2d 1881 (S.D. Ind.1993). Also, the identical invention must be described or shown in as complete detail as is contained in the claim. *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989); *Chester v. Miller*, 15 USPQ2d 1333 (Fed. Cir. 1990); M.P.E.P. § 2131.

The Office alleges that Langmann et al. anticipates or obviates claim 3 and the dependent claims because it discloses a polynucleotide that encodes a polypeptide which is more than 98% identical to SEQ ID NO: 2. Applicants appreciate the facsimile copy of the Blast Sequence Alignment Report that the Examiner forwarded on August 29, 2003. However, Applicants respectfully submit that the incorrect sequence was used for the sequence alignment generated by this report. Specifically, Applicants submit that the amino acid sequence used to generate the sequence alignment is not the amino acid sequence taught by Langmann et al. The first twenty amino acids of the sequence disclosed in the Langmann reference (BBRC, April 1999, 257(1):29-33), GenBank

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Accession No. AJ012376, and Swiss Protein 095477 are: MPSAGTLPWVQGIICNANNP (see attached Gen Bank report for AJ012376, which references BBRC, April 1999, 257(1):29-33 and Swiss Protein: 095477; Exhibit 2). However, the first 20 amino acids for the sequence used in the report are: MACWPQLRLLLWKNLTFRRR (see page 3 of the report attached herewith; Exhibit 2).

The Office rejects claim 3 apparently for reciting a polynucleotide that encodes a polypeptide which is more than 98% identical to SEQ ID NO: 2 and does not address the other polynucleotides listed in claim 3. Despite the lack of reasoned rejection for the other polynucleotides recited in original claim 3, Applicants provide the following brief comments.

The sequence disclosed in Langmann et al. (and the corresponding Swiss Protein Accession No. 095477 and GenBank Accession No. AJ012376) does not anticipate claims 77-80. Langmann et al. discloses an ABC1 polypeptide of **2201 amino acids** and a corresponding **6880 bp polynucleotide** having a **6603 bp open reading frame**. In contrast, claim 77 is directed to an isolated polynucleotide having ABC1 activity comprising SEQ ID NO: 1, which is a **10,442 bp polynucleotide** having a **6783 bp open reading frame**. Given that Langmann et al. teaches a polynucleotide having only 6880 nucleotides, it does not anticipate claim 77. Further, the disclosure of Langmann et al. does not render claim 77 obvious. Langmann only teaches a 6783 bp polynucleotide that does not even include the entire coding region of ABC1. As taught in the instant application, the additional nucleotides found in SEQ ID NO: 1 constitute sequences located at the 5' end of the coding region, as well as sequences in the 5' and 3' untranslated regions. As provided in the specification at, e.g., pages 32-33 and Examples 17 and 18, these 5' and 3' end sequences contain regulatory sequences of the ABC1 gene. As known in the art, general regulatory sequences in the 5' untranslated region are critical for initiation of transcription (i.e., SP1 sites and TATA box) and initiation of translation of ABC1. Further, as taught in the specification at pages 88-95 and Figure 13, the 5' untranslated region also contains binding sites for transcription factors with roles in lipid metabolism (i.e., LXR and sterol response elements). Thus, the newly described 5' nucleotides found in SEQ ID NO: 1 not only contain coding region sequences, but further

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contain gene-specific regulatory sequences directly involved in the regulation of ABC1 gene expression and cholesterol transport. In addition, as known generally in the art and further taught in the specification at page 33, the sequences in the 3' untranslated region contain sequences important for transcript stability. Given that the newly described 5' and 3' end regulatory sequences found in SEQ ID NO: 1 affect the level and stability of the ABC1 transcript, and consequently the level of ABC1 protein, they play an important functional role in cholesterol transport. These sequences are not disclosed in the Langmann et al. reference; indeed, the Langmann et al. reference would lead one of ordinary skill to believe that such sequences were *not* a part of the ABC1 cDNA. Accordingly, Langmann et al. does not obviate the polynucleotide recited in claim 77.

Nor does Langmann et al. anticipate or obviate claims 5 and 6, which are directed to an isolated polynucleotide having ABC1 activity comprising a nucleotide sequence that has at least 90% identity or 95% identity with the polynucleotide comprising SEQ ID NO: 1, respectively. Assuming 100% nucleotide sequence identity between the 6880 bp polynucleotide disclosed by Langmann et al. and SEQ ID NO: 1, a polynucleotide sequence that is 6880 bp has, at best, only 66% identity with SEQ ID NO: 1, which is clearly outside the scope of the claims.

Claim 78 is directed to a polynucleotide encoding a polypeptide comprising **2261 amino acids** (SEQ ID NO: 2). Given that Langmann et al. teaches a polypeptide having only **2201 amino acids** (i.e., lacking 60 amino acid residues found at the N-terminus of SEQ ID NO: 2), it does not anticipate claim 78. Further, the disclosure of Langmann et al. does not render claim 78 obvious. Langmann et al. clearly teaches an ABC1 protein having only 2201 amino acids and does not even contemplate an ABC1 protein having a greater number of amino acids, i.e., 2261 amino acids. In fact, by teaching the incorrect methionine start site and by teaching a 2201 amino acid ABC1 protein, the Langmann et al. reference teaches away from the presently claimed polynucleotide encoding a polypeptide of **2261 amino acids** (SEQ ID NO: 2)

Claim 79 is directed to an isolated polynucleotide comprising nucleotides 291-7074 of SEQ ID NO: 1. The sequence containing nucleotides 291-7074 of SEQ ID NO: 1 corresponds to the full-length open reading frame sequence of ABC1 (6783 bp).

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Langmann discloses a polynucleotide sequence having an open reading frame of only 6603 bp. Due to the failure to recognize that the open reading frame is actually 6783 bp in length, the Langmann et al. reference fails to identify 180 bp of the 5' end of the open reading frame and does not even disclose the terminal 60 bp located at the 5' end of the open reading frame. Accordingly, the sequence disclosed in Langmann et al and GenBank Accession No.AJ012376 does not disclose a polynucleotide sequence that contains nucleotides 291-7074 and thus fails to anticipate claim 79. Further, the disclosure of Langmann et al. does not render claim 79 obvious. Langmann et al. clearly teaches a polynucleotide sequence having an open reading frame of only 6603 bp and does not even contemplate a polynucleotide sequence having a longer open reading frame, i.e., 6783 bp. In fact, by teaching the incorrect open reading frame and by teaching an open reading frame of 6603 bp, the Langmann et al. reference teaches away from the presently claimed polynucleotide comprising nucleotides 291-7074 of SEQ ID NO: 1.

Claim 80 is directed to an isolated polynucleotide encoding a polypeptide having at least 98% sequence identity with SEQ ID NO: 2. The polypeptide disclosed in Langmann et al. has 2201 amino acids. The polypeptide of SEQ ID NO: 2 has 2261 amino acids. Assuming 100% amino acid sequence identity, a polypeptide sequence that is 2201 amino acids in length has, at best, only 97% identity with SEQ ID NO: 2. Further, there are at least 7 amino acid differences between the Langmannn et al. sequence and SEQ ID NO: 2 as taught in the specification at page 30. Thus, the 2201 amino acid polypeptide disclosed in Langmann et al. is not a polypeptide having at least 98% sequence identity with SEQ ID NO: 2, as required by claim 80. Given that the 2201 amino acid sequence taught in Langmann et al. does not teach each and every element as set forth in the claim, it does not anticipate claim 80. Further, given that Langmann et al. clearly teaches an ABC1 polypeptide having only 2201 amino acids and does not even contemplate an ABC1 polypeptide having a greater number of amino acids, i.e., 2261 amino acids, Langmann et al. does not teach or suggest the polypeptide of SEQ ID NO: 2 (i.e., polypeptide having 2262 amino acids); nor does it teach or suggest a polypeptide having at least 98% sequence identity with SEQ ID NO: 2.

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The Office further alleges that the dependent claims are obvious over Langmann et al. However, given that the disclosure of Langmann et al. does not render claims 77-80 (i.e., corresponding to original claims 3) or claims 5 and 6 obvious for the reasons stated herein, Langmann et al. does not render obvious the claims dependent thereon.

Applicants respectfully request withdrawal of the 102(b)/103(a) rejection based on Langmann et al. and GenBank Accession No. AJ012376.

35 U.S.C. § 102(e)/103(a) Rejection in view of Rosier-Montus et al.

The Office rejected claims 3-24 and 33 under 35 U.S.C. § 102(e) as allegedly being anticipated by or, in the alternative, under 35 U.S.C. § 103(a) as allegedly obvious over Rosier-Montus et al. (US 20020146792A1). The rejection is respectfully traversed.

Rosier-Montus et al. was published on October 10, 2002, with a priority date of May 2, 2000. Applicants submit herewith an Inventor Declaration under 37 CFR §1.131 to establish a date of invention prior to the May 2, 2000 priority date of Rosier-Montus. See Exhibit 3, Declaration of Dr. Richard Lawn, and the supporting Exhibits A-D. Given that the inventions claimed in the present application were invented prior to May 2, 2000, Rosier-Montus is not applicable prior art.

Accordingly, Applicants respectfully request withdrawal of the 35 U.S.C. § 102(e)/103(a) rejections in view of the Rosier-Montus reference.

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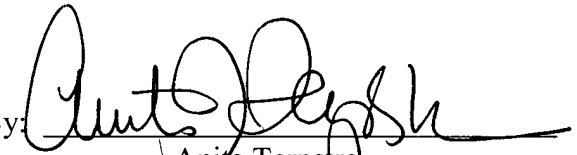
Conclusion

In view of the above remarks, the application is considered to be in good and proper form for allowance and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of this application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,
McDonnell Boehnen Hulbert & Berghoff

Date: October 1, 2003

By: 
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Review

The ABCA subclass of mammalian transporters

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Abstract

We describe here a subclass of mammalian ABC transporters, the ABCA subfamily. This is a unique group that, in contrast to any other human ABC transporters, lacks a structural counterpart in yeast. The structural hallmark of the ABCA subfamily is the presence of a stretch of hydrophobic amino acids thought to span the membrane within the putative regulatory (R) domain. As for today, four ABCA transporters have been fully characterised but 11 ABCA-encoding genes have been identified. ABCA-specific motifs in the nucleotide binding folds can be detected when analysing the conserved sequences among the different members. These motifs may reveal functional constraints exclusive to this group of ABC transporters.

Keywords: ABCA subclass; ABC1; ABCR; Consensus motif; Gene family

Contents

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1. Introduction

The family of ABC transporters is one of the largest family of proteins. In genomes whose sequence has been completed they represent between 2–5% of the whole coding regions [1–4] An arbitrary extrapolation to the human genome which is estimated to contain 60–70 000 genes, will lead to an expected number of ABC transporters of more than 1000, which is likely to be by far an overestimation. Since a comprehensive list is present in the ABC transporter web page (http://www.med.rug.nl/mdl/humanabc.htm), and the ABC transporters in various organisms are reviewed in detail in this special issue, we concentrate here only on the ABCA subfamily.

The structural archetype of a full size ABC transporters encoded by a single gene is the P-glycoprotein which possesses a canonical 6×2 transmembrane spanners paired in tandem to the nucleotide binding domains (NBD). However, several subfamilies can be identified on the basis of structural features. Our aim here is to discuss the distinctive structural features of the ABCA subfamily, which appears to be restricted to multicellular organisms. Indeed, the class of mammalian ABC1-like proteins, or, according to the novel nomenclature proposed by the genome project ABCA proteins, lacks a yeast structural ortholog [5–7]. Interestingly, however, transporters sharing similar features can be found in other evolutionarily distant organisms (see below).

2. Features of the ABCA class of ABC transporters

When analysing the prototype of this class, ABCA1 (formerly ABC1), we reported the presence of a regulatory domain reminiscent of the R domain in CFTR. However, here this domain is split into two halves by a highly hydrophobic segment, which we called HH1 [8]. Based on secondary structure predictions, which predicted HH1 as a transmembrane segment but favoured a β conformation, and from analogy to similarly structured domains in K^+ channels [8–11], we proposed the topological model shown in Fig. 1, panel A. The experimental support to this model comes from the analysis of the protease sensitivity of a number of Myc-tagged ABC1 chimeras, transcripted and translated in vitro in the presence of microsomal membranes (Hamon and Chimini, unpublished) and immunoprecipitated with the anti-myc monoclonal antibody 9E10 [12]. The validity of the model was also examined by a specific antibody reacting with the first NBD of ABC1 [13]. These experiments allowed to define the position of the extracellular loop between TM5 and TM6, which contains a glycosylated asparagine, to confirm the intracellular location of the two ABC domains, as well as the cytosolic location of the second half of the regulatory domain. However, as it is frequently the case with polytopic membrane proteins, alternative topologies cannot be excluded. An example is shown in Fig. 1, panel B, where the HH1 domain is represented as spanning the membrane once from inside to outside. This prediction includes a large extracellular loop of 260 residues, corresponding in the former model to the second half of the R domain. We are currently testing these models by analyzing the reactivity of surface-exposed transporters with a panel of antisera, generated against predicted extracellular loops. For the following discussion we will refer to the topology model shown in panel A.

3. The ABCA gene family

In 1994 we reported the identification of ABCA1 and its structural peculiarities among ABC transporters [8]. It soon became apparent that ABC1 was not a unique example and a large group of transporters shared similar features [8,14–17] (Table 1). Indeed, so far four ABCA genes have been fully sequenced, namely ABCA1, ABCA2, ABCA3 or C and ABCR, either in mouse, man or other mammals. We have recently identified a fifth transporter conserved in mouse and man (tentatively called ABCA7 or ABCX (Broccardo et al. submitted and Dean and Allikments, personal communication). and have evidence for the presence of at least five other individual ABCA genes. Recently, the analysis of osteoclast-specific transcripts has also allowed the identifications of partial sequences (named OCA), closely related to the ABCA family (Wagstaff, personal communication). Their relationship to known ABCA transporters, however, is still unknown.

A pairwise comparison of the published full nucleotide sequences for ABCA cDNAs shows an over-

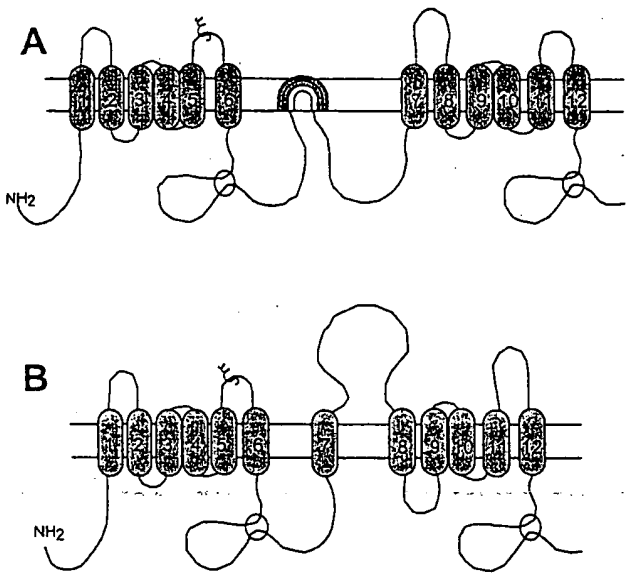

Fig. 1. Predictive topology of ABCA1 as a prototype of the ABCA subclass of transporters. (A) Topological model proposed by the authors with two symmetrical halves interrupted by an R-domain. The highly hydrophobic segment (HH1) is drawn as a hairpin crossing the membrane twice. Each half is composed of a set of six transmembrane spanners and an ATP binding cassette. (B) Alternative topological prediction, with HH1 spanning the membrane once, from the inside to the outside. This leads to an asymmetric structure with a large extracellular loop followed by an odd number of transmembrane spanners in the second half of the transporter.

all identity of around 60% among the different genes, irrespectively of the pairs analysed. Cross-species conservation of individual genes is extremely high, exceeding 85% identity. This allows to design member-specific probes, detecting unique transcripts in Northern blots (Fig. 2). Indeed, although the length of each transcript is around 7 kb, slight differences in size can be seen when the RNAs are separated on agarose gels, as shown in panel A. In addition, when a panel of tissues and cell lines is used to hybridise, all these genes appear to be expressed ubiquitously at low levels. Still, a preferential and non-redundant territory of expression can be assigned to individual members. ABCA1 is 10-fold overexpressed in the uterus of pregnant women [8], followed by the expression levels in liver, adrenals and normal uterus, whereas ABCA2 is overexpressed in the adult brain, and ABCA3 in the liver, lung and kidney (Fig. 2). The most recently recognised member, ABCA7 or ABCX, is preferentially expressed in the spleen and more generally in lymphoid organs (not shown, Broccardo et al., in preparation). ABCR was not included in this analysis, since its expression has been reported to be restricted to the retina [18,19].

The currently estimated number of ABCA genes is at least 11, based on the analysis of dbEST by Dean and co-workers (personal communication). A similar estimation was obtained in our laboratory, by a genomic amplification assay with degenerate primers targeting ABCA-specific consensus motifs, which flank conserved introns in the NBDs (see below and Broccardo et al., unpublished observation).

All the fully identified ABCA genes map in synthenic regions in the mouse and human genome, respectively, and there is no evidence of gene clustering (Table 1). This suggests an evolutionary origin of

Table 1
The family of ABCA transporters

Name	Symbol	Chromosome human	Chromosome mouse	RNA	Amino acid	Exons	Accession
ABC1	ABCA1	9q22-q31	4A5-B3	6.9	2259	48	AJ012376
ABC2	ABCA2	9q34	2A2-B	6.7	>2174	>42	U18235
ABC3 (ABC-C)	ABCA3	16p13.3	17B	6.5	1704	?	U78735
ABCR	ABCA4	1p22	?	7.3	2273	50	NM000350
ABC4	ABCA7	19p13.3	10B4-C1	6.6	>1840	>37	-
EST90625	ABCA5	17q21-q24	?	?	?	38	U66672
EST155051	ABCA6	17q21	?	7	?	38	U66680
KIAA0822	ABCA8	17q24	?	5.7	1581	38	AB020629
EST640918	ABCA9	17q24	?	?	?	38	-
EST698739	ABCA10	17q24	?	?	?	38	-
EST1133530	ABCA11	4p16-pter	?	?	?	?	-

For yet unpublished results the sources are Broccardo et al., (ABCA7) and Dean et al. (ABCA5-6 and 8-11). KIAA0822 [45]

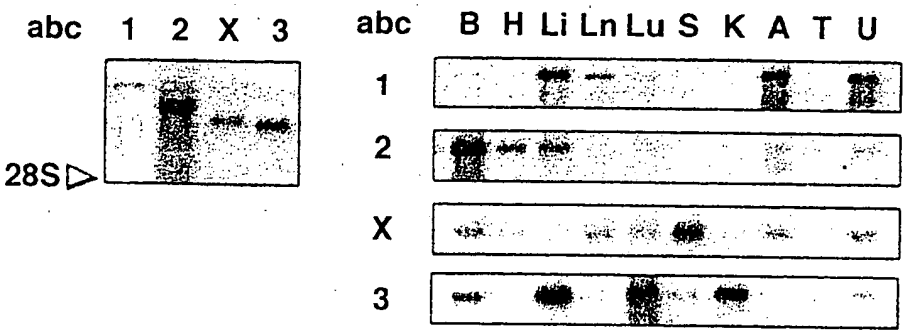

Fig. 2. Expression of ABCA transcripts. (A) Northern blot analysis of brain RNA with ABCA member-specific probes highlights small, but detectable differences in size. ABCA1 transcript is the largest one, followed by ABCA2, ABCA7 and ABCA3. (B) Northern blot analysis of a panel of RNAs from adult mouse tissues, hybridised with member-specific probes shows that all ABCA transcripts are detected ubiquitously at low levels. Non-redundant member-specific territories of preferential expression are nonetheless detectable. 1: ABCA1, 2: ABCA2, X: ABCA7, 3: ABCA3, B: brain, H: heart, Li: liver, Ln: lymph nodes, Lu: lung, K: kidney, A: adrenals, T: thymus, U: uterus.

individual members, predating speciation. Dean and co-workers have recently identified an additional ABCA gene on chromosome 4p16-pter, and a cluster of tandemly linked ABC genes on chromosome 17q24, apparently sharing similarities to the ABCA class (Dean, personal communication). A more detailed analysis and a final hypothesis about their evolutionary relationships will have to wait for the complete sequences and the mapping of the orthologs in the mouse genome.

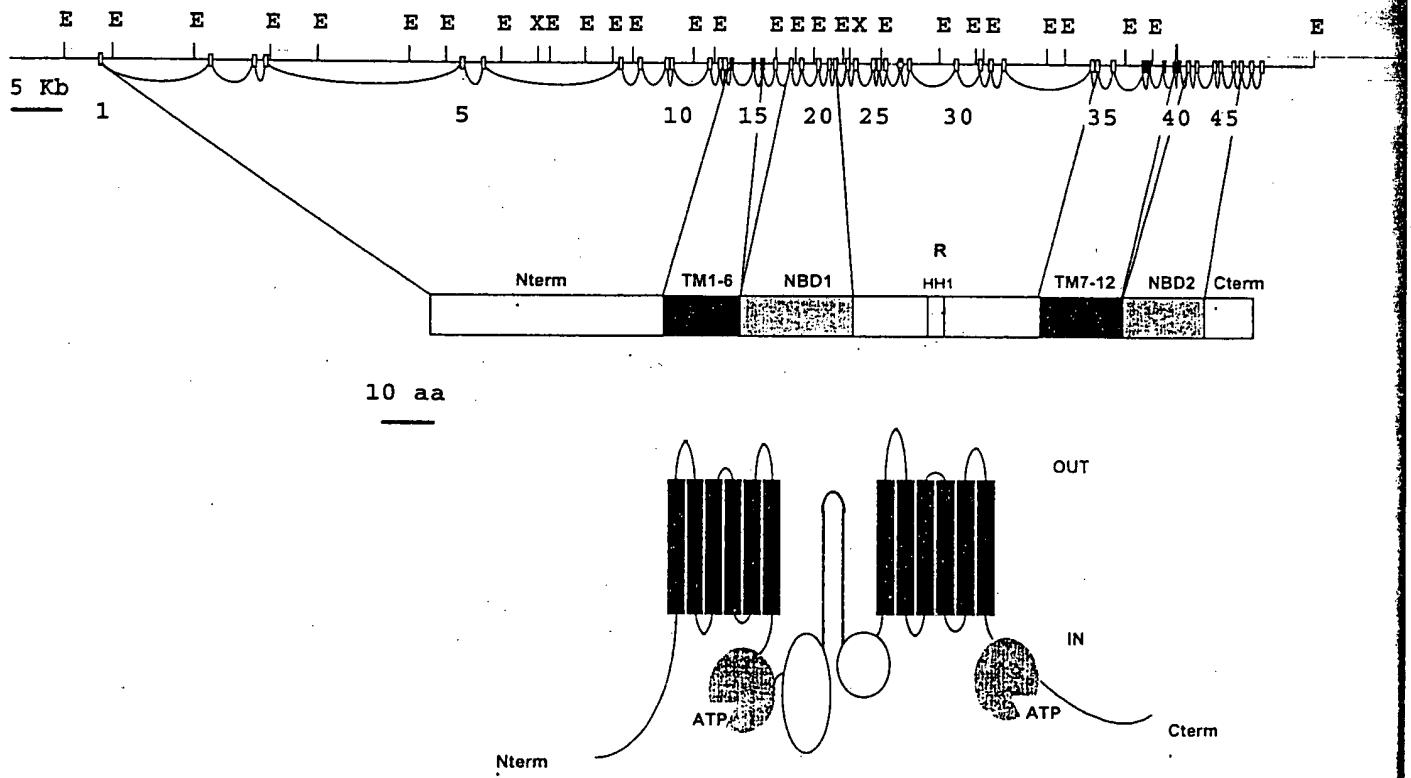

Fig. 3. Correspondence between genomic structure of the ABCA1 gene and protein domains. (Top panel) Exon-intron structure of the ABCA1 gene on mouse chromosome 4. Exons are shown as filled boxes. The positions of *Eco*RI (E) and *Xho*I (X) restriction sites are given. (Middle and bottom panel) Exon-encoded protein domains are highlighted by graphic motifs, either in the linear representation or in the predicted protein. TM: transmembrane helix, NBD: nucleotide binding domain, R: regulatory domain.

4. Gene organisation

A full genomic organisation has been published for human ABCR [20,21]. That of the mouse ABCA1 gene is schematically shown in Fig. 3 and has been deposited in the databases. The mouse ABCA1 gene spans more than 100 kb on chromosome 4A5-B3, and is split into 49 exons (Broccardo et al., personal communication). The overall exon organisation and the correspondence between exons and the encoded protein domains are shown in Fig. 3. It has to be noted that a striking conservation of the gene structure organisation is present among the different members of the ABCA subclass. Most of the splice junctions are indeed perfectly conserved among the different genes, for instance 42 out of the 48 introns of ABCA1 are perfectly conserved in ABCR, both in position and type [20]. No clusters of introns of the same type can be evidenced, however, in any of the analysed genes.

A cross-gene analysis of the intron-exon organisation reveals the highest conservation among members in the regions encoding the NBDs and the transmembrane anchors (Fig. 4 and 5). In contrast, a comparison of the structure of the N- and C-terminal halves of a single gene shows that equivalent domains do not share a similar genomic organisation. This suggests an independent evolution, which is not unusual among ABC transporters. It does once again corroborate the evolutionary hypothesis of the origin of multidomain transporters from fusion of independently evolved individual domains, as opposed to internal duplication events giving rise to an ancestral transporter. A comparison of the structural organisation of the ABCA gene subclass with other structural classes, like MDR, MRP, CFTR or SUR transporters, shows class-specific diversifications and, again, corroborates the autonomous evolutionary history of each class of genes [22–26].

Finally, and in contrast to the NBD and TD encoding regions, the genomic fragment encoding the regulatory domain shows an extreme variability in the positions of splicing sites among individual transporters and in the number of exons (10 exons in ABCA2 and ABCA7, 13 for ABCA1 and ABCR). This variability is reflected down to the amino acid level, since this domain shows the least conservation in primary sequence. Its main characteristics, however, like the richness in charged residues, the presence of several putative phosphorylation sites, and the presence of the highly hydrophobic spanner, are constantly present. In spite of the invariable detec-

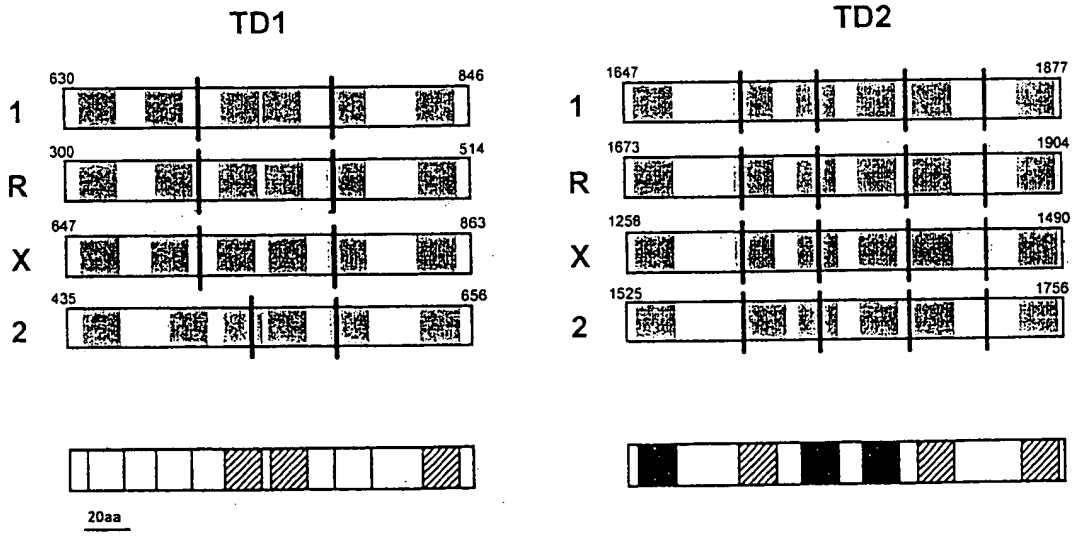

Fig. 4. Conservation of the gene structure across four different ABCA transporters. Transmembrane Domains (TD1 = N-terminal, TD2 = C-terminal). The amino acid positions, taken as borders, are indicated. For simplicity, the limit of each domain corresponds to the limits of the exons. The predicted TM spanners are shown in grey. The bottom rectangle shows the percentage of sequence identity across members for each spanner. Symbols: black solid square: 40–60%, thick hatched squares: 20–40%, thin hatched squares: 0–20% of identity. Exon-introns boundaries are shown by vertical bars. 1: ABCA1, 2: ABCA2, X: ABCA7, R: ABCAR.

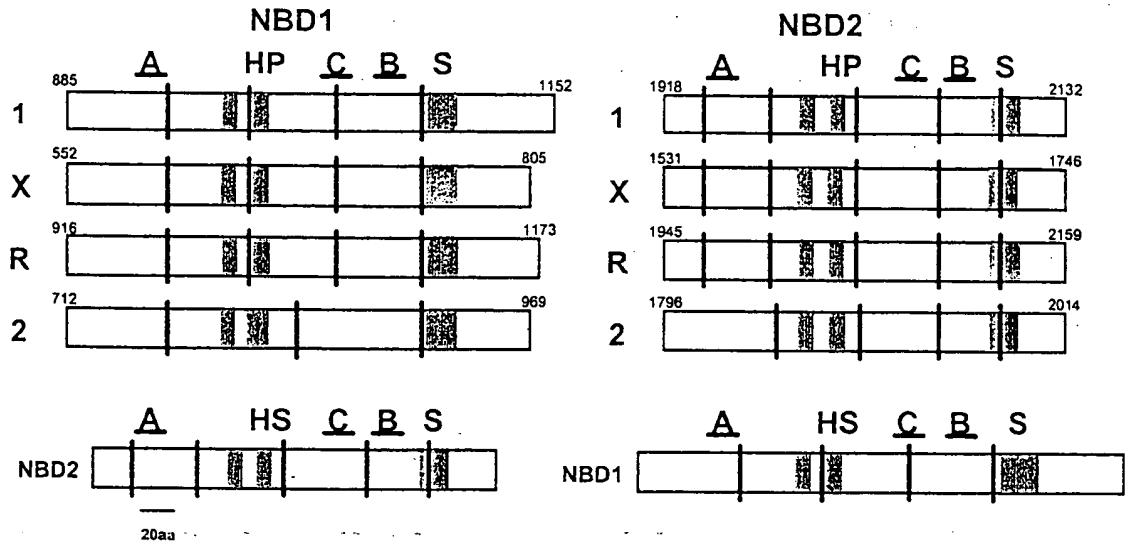

Fig. 5. Conservation of the gene structure across four different ABCA transporters. (NBD1 = N-terminal, NBD2 = C-terminal). The comparative genomic structures of four ABCA transporters are shown. The boundaries of the domains are indicated and correspond to the exons of interest. Introns are indicated by black bars. Conserved motifs are indicated by shaded bars. HS: hot spot, S: switch region, A: Walker A, B: Walker B, C: active transport signature. The bottom rectangle shows for comparison the organisation of a typical ABCA NBD2 or NBD1. 1: ABCA1, 2: ABCA2, X: ABCA7, R: ABCR.

tion of HH1, its position varies largely in each member; this leads to member-specific configurations of the R domain.

5. Sequence conservation and ABCA signatures

At the amino acid level, the known ABCA transporters show an identity from 45–66% along the whole sequence. The comparative analysis of their hydrophobicity plots also reveals highly similar profiles, leading to close, but unfortunately, still ambiguous predictions of topology.

For the sake of simplicity, the following discussion on ABCA sequence conservation will be articulated according to a conventional segmentation in domains. The border of each domain has been fixed according to exon limits. We define TD as the group of transmembrane helices and their intervening loops. This corresponds in ABCA1 to residues 630–846 for the N terminal TM 1–6 (exon 14–16) and from 1647–1877 for the C terminal set of membrane spanners (TM 7–12; exon 36–40). In the figure presented we label these limits for each of the analysed transporters.

An analysis of the sequences of TDs across the individual ABCAs demonstrates a higher degree of conservation in the second set of spanners (illustrated schematically in Fig. 4), in spite of a relatively low degree of primary sequence conservation, compared to that in the NBDs (average identity score of 44% for TD1 and 53% for TD2). This is particularly strik-

Table 2
ABCA specific signatures can be highlighted in the extended NBDs

	Walker A - #1	#50	Hot spot	ATS/C	Walker B	Switch #162
NBD1	GQ••••LGHNGAGKTTT	G•CPQ•N	LTV•EH••FY	LSGGM•RK	LDEPT•G•DP	L•TH•MDEA••LGDR
NBD2	GECFGLLGVNGAGKSTT	GYCPQFD	LTGRE•L	YSGG•KRK	LDEPTTGMDP	LTSHSMEECEALC•R

For simplicity the position of Walker A is numbered as +1. Residues in bold are conserved in all subclasses of the ABC transporter family. Normal face residues are conserved at 80% in ABCAs, whereas • corresponds to variable residues. NBD1 or NBD2 corresponds to the N or C-terminal NBD.

ing for the TM VII, IX and X, which all show identities higher than 50%. In addition, according to model A, the first and last extracellular loops in both sets of transmembrane regions show a divergent behaviour in term of sequence conservation. Whereas the 21 amino acid long loop between transmembrane helices I-II and the 36 amino acid long loop between TM VII-VIII are extremely conserved, the last extracellular loop (V-VI; 26 amino acids and XI-XII; 32 amino acids) can be considered hypervariable among the individual transporters.

The NBD corresponds to the extended nucleotide binding domain, i.e. in ABCA1 it spans from amino acid 885–1152 for the N-terminal one (NBD1: exon 18–22) and 1918–2132 for the C-terminal one (NBD2, exon 42–47). An overall analysis of sequence conservation at the NBDs shows an identity from 55 to 61% (average 59%) for NBD1, which increases to 57–69% (average 65%) at the NBD2. As already noted for the exon organisation, an intramolecular comparison of the two NBDs leads to a lesser degree of conservation, with an average value of 34,4%. This is definitely higher than the identity score between corresponding NBDs in ABC transporters belonging to different structural classes. These values range from 21 to 26% when comparing CFTR, MDR or TAP NDBs with the corresponding domains in ABCA1. A comparison of the two NBDs in other ABCs leads to variable results. For instance, in the case of CFTR, the identity score is 24%, while for MDRs and the two TAPS the score is 57 or 55%, respectively.

A closer analysis of NBD conservation leads to the definition of motifs unique to the ABCA family. These are summarised in Table 2 and detailed below. As required to belong to the family of ABC transporters, all ABCA proteins show in the NBDs the classical consensus Walker A, B and C, or ATS motifs (see [27]). Nonetheless, as it is the case for the different structural subgroups in yeast, specific signatures can be highlighted. First, at the level of the Walker motifs, a high conservation of residues surrounding either the conserved glycines in Walker A or the aspartate in Walker B can be documented. It is of note that similar conservations are found both in NBD1 and NBD2.

At least three other ABCA motifs can be defined. The so called 'switch' region, around the key histidine residue, downstream to Walker B, is highly conserved. Similarly, the cluster of residues surrounding the glutamine +50 from Walker A, and the 'hot spot' region, that roughly corresponds to the position of DF508 in CFTR [6,27,28] are conserved. In ABCAs the histidine is close to a highly acidic sequence, whereas the glutamine is invariably preceded by the doublet cysteine-proline. In the MDR/TAP subfamily there is no evidence for an acidic stretch of residues nearby the histidine, whereas both the histidine and the charged stretch are lacking in the CFTR/MRP cluster. All these consensus motifs are detectable in both NBDs, in spite of minor differences. An additional characteristic of the ABCA proteins is the symmetrical presence, downstream to both NBDs (+100–130 amino acids from Walker B), a conserved stretch of amino acids (T • EE•FL • V•E for the NBD1 and D•SV•Q• •L E/D N/Q VF), whose significance is so far unknown.

6. ABCAs in invertebrates

In order to estimate the reliability of the above described motifs, we scanned the ABC sequences in the databases from different genomes. This resulted in the identification of potential ABCA transporters in plants, insects and lower organisms. For instance, in *Arabidopsis thaliana*, several sequences encoding partial ABCA transporters could be detected. After eliminating redundancy and overlapping clones, we concluded that at least two full size ABCA sequences have been already sequenced in *Arabidopsis*, namely AC002339 in chromosome II, and AL049746 on chromosome III. Similarly, in *Dictyostelium discoideum*, the abcA sequence (U66526) shows high similarity to the ABCA subfamily, and again, all the above described motifs are present. In contrast to the mammalian ABCAs, however, AbcA possesses a unique NBD. Three ABCA full size transporters can be found in the *Caenorhabditis elegans* genome. One corresponds to ced-7 [29], which can be considered the functional ortholog of ABC1 in the nematode (see below), the two others share an identity higher than 40% at the amino acid level and correspond to AF10131 and AF003146 [30]. From a scan of the *Drosophila* genome project, three genomic sequences, potentially translating into ABCA trans-

porters with a full-size multidomain structure, were detected (AF034856, AC004321, AC004348). Preliminary information from F. Gamarro (personal communication) indicates that ABCA-encoding sequences, identifiable by the presence of these diagnostic motifs, exist also in parasites like *Trypanosoma cruzi* and *Leishmania tropica*.

7. ABCA1 and ced-7: the engulfment of apoptotic corpses

Among the members of the ABCA subgroup of transporters, ABCA1 has been functionally associated to the process of the engulfment of cells dying by apoptosis [13]. Several lines of evidence support the hypothesis. The first derives from the analysis of the ABCA1 expression pattern during mouse embryonic development. This analysis showed a direct spatio-temporal relationship between the expression of ABCA1 transcript and the areas of programmed cell death. In these areas the locally recruited macrophages, known to express ABC1, are actively engaged in the clearance of cell corpses. Consistently, in an 'in vitro' situation, the inhibition of ABCA1 function by a specific antibody greatly reduces the ability of peritoneal macrophages to phagocytose apoptotic thymocytes. This antibody has no effect on their ingestion of yeast, thus functionally linking ABC1 to the process of apoptotic engulfment. Last but not least comes the recent demonstration that an ABCA transporter of *C. elegans*, sharing around 30% identity with ABCA1, is able to complement ced-7 mutants [29]. Ced-7 belongs to one of the two epistatic groups of genes controlling the clearance of corpses generated by cell death during the development of the nematode. Altogether, these results strongly suggest that ABC1 and ced-7 are orthologs in these two evolutionary distant species.

It has to be noted, however, that although the similarities between ced-7 and ABC1 are quite high, in the absence of functional data it would have been extremely difficult to select the best candidate as an ortholog among the mammalian members of the ABCA class of transporters. Indeed, an exclusive analysis of sequence comparisons reveals roughly equivalent identities, ranging from 27 to 32% between ced-7 and each of the individual ABCA transporters. The precise molecular function exerted by an ABC transporter during the engulfment is, however, still an open question. Recent and preliminary results suggest an involvement of ABCA1 in the control of membrane lipid composition. Indeed, both in ABCA1 null mice and in overexpressing transfectants, an altered membrane mobility of phospholipids can be assessed (Broccardo et al., submitted). This might well fit the recently proposed concept that the engulfment of apoptotic bodies is an exquisite form of phagocytosis, since it requires the transbilayer movements of lipids on the surface of the phagocytes (Marguet et al., in press). Along the same line, mutations in the human gene encoding ABCA1 have been detected in patients affected by Tangier disease [31–34], a rare autosomal recessive disorder of lipid metabolism [35,36] whose locus indeed has been mapped to human chromosome 9q31 [37].

8. ABCR and chorioretinal degeneration

Frequently ABC transporters have been associated to diseases. In the group of ABCAs, so far only ABCR has been associated unambiguously to the pathogenesis of degenerative eye diseases [38–40]. In fact it has been reported by several groups that mutations in the ABCR coding regions can be detected in a large panel of degenerative illnesses of the retina, from Stargardt disease to recessive retinitis pigmentosa or cone-rod dystrophy [19,38,41]. A thorough discussion on the complex genetics of ABCR mutations and eye diseases is out of the scope of this article and can be found in [38]. No evidence on the molecular function of ABCR as a transporter has been provided, with the exception of the report that retinal activates its ATPase activity [42]. This makes retinal a good candidate as a substrate for ABCR-mediated transport, in analogy with the case of P-gp, whose enzymatic function can be stimulated by specific substrates [42–44].

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Cholesterol efflux regulatory protein, Tangier disease and familial high-density lipoprotein deficiency

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Cellular cholesterol efflux, by which cholesterol is transported from peripheral cells to HDL acceptor molecules for transport to the liver, is the first step of reverse cholesterol transport. Two genetic disorders, Tangier disease and some cases of familial HDL deficiency, have defects of cellular cholesterol efflux. The recent discovery of mutations in the *ABC1* gene, which encodes the cholesterol efflux regulatory protein, in both these disorders establishes cholesterol efflux regulatory protein as a rate-limiting factor in reverse cholesterol transport. Curr Opin Lipidol 11:117–122.

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Abbreviations

CERP	cholesterol efflux regulatory protein
FHA	familial HDL deficiency
MMP	matrix metalloproteinase
RCT	reverse cholesterol transport
SR-BI	scavenger receptor class B, type I

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Introduction

Intracellular cholesterol homeostasis in cells is exquisitely regulated and dependent on the balance between cholesterol synthesis and influx, its degradation, and cholesterol ester formation, translocation to the plasma membrane for efflux, and transport from the peripheral cell to the liver. Humans synthesize and degrade approximately 1 g cholesterol/day. About two-thirds of cholesterol is transported on LDL particles and approximately one-third of that cholesterol is deposited into extrahepatic tissues.

This concept of reverse cholesterol transport (RCT) was first proposed by Glomset in 1968 [1]. The efflux of cholesterol from cells represents the first step of RCT and can operate through a variety of mechanisms. Simple passive diffusion occurs when cholesterol is desorbed from the plasma membrane down the concentration gradient into the aqueous phase, and incorporated into phospholipid-containing acceptor particles. Active transport is a rate-limiting mechanism for removal of cholesterol from cells. In this process cellular cholesterol is transported to lipid depleted apolipoproteins [2]. In addition, scavenger receptor class B, type I (SR-BI)-mediated cholesterol exchange influences cholesterol flow into and out of specific cells. Interestingly, the mechanism by which SR-BI affects cholesterol efflux is not related to its ability to bind to a lipoprotein cholesterol acceptor. Cholesterol transport to phospholipid vesicles or cyclodextrins (neither of which binds to SR-BI) is also enhanced by SR-BI. There is some evidence that SR-BI alters the distribution of cholesterol in the cell, presumably making it more available for efflux (for review [3]). The rate of cholesterol efflux varies between cell types and is also influenced by the nature of the acceptor particle, with levels of HDL-phospholipids as major determinants of cholesterol efflux [4–9].

Recently, we and others [10••–12••] have identified the *ABC1* gene as a key determinant of active transport of cholesterol and phospholipids in certain cells. The clinical manifestations of Tangier disease with cholesterol ester accumulation in specific cell types, associated with premature coronary artery disease in some carriers for mutations, identifies the protein encoded by *ABC1*,

cholesterol efflux regulatory protein (CERP), as an important determinant of HDL levels in humans and an important component of the RCT pathway.

New insights concerning factors that influence cholesterol efflux from cells

Cells have evolved tightly regulated mechanisms for control of cholesterol concentrations. As cholesterol increases in cells, it may be stored in an acylated form produced by the action of acyl-coenzyme A-cholesterol-acyltransferases 1 and 2. Apolipoprotein AI can mobilize this stored cholesterol. Recent evidence has suggested that particular domains in apolipoprotein AI influence cholesterol efflux from cells. The carboxyl-terminal region of apolipoprotein AI encompassing residues 209–243 are important in promoting cholesterol efflux from cells [13,14], whereas apolipoprotein AI residues 143–164 appear to be crucial for lecithin:cholesterol acyltransferase activation [15–16].

For active transport of cholesterol from cells there is a need for appropriate ATP-dependent transfer of cholesterol and phospholipid, and also the requirement for an intact receptor molecule [17]. Further evidence for the importance of the carboxy terminus of apolipoprotein AI in mediating cholesterol efflux has been derived from studies of the influence of matrix metalloproteinases (MMPs), which reduce HDL-induced cholesterol efflux from human macrophage foam cells. The ability of several members of the MMP family to degrade human HDL and dramatically decrease cholesterol efflux highlights the importance of the integrity of the acceptors of cellular cholesterol and their role in cholesterol efflux [18•]. This is consistent with studies [19] that show that trypsin treatment of HDL can abolish apolipoprotein-mediated cholesterol removal. The proteolytic modification of apolipoprotein AI by MMPs, and possibly by other as yet unknown molecules, may be crucial in blocking the physiological role of apolipoprotein AI in RCT from human atherosclerotic lesions. Further evidence of the importance of the availability of a lipid-poor apolipoprotein acceptor molecule comes from a recent study [20] that showed that the phospholipid transfer protein may aid in the generation of these acceptors and thereby enhance efflux.

It has previously been demonstrated that oxidative modification of LDL and its consequent accelerated uptake by arterial wall macrophages are important in the development of atherosclerosis. Similarly, HDL that contain high levels of polyunsaturated fatty acids (which can also be oxidized) are less effective in promoting cholesterol efflux from macrophage-derived foam cells. In contrast to oxidation, glycation of HDL appears to have no obvious effect on the capacity of HDL to mediate cholesterol efflux [21].

In addition to the extracellular cholesterol acceptor, cell type and intracellular environment may also influence cholesterol efflux [3••]. Metabolic manipulation of cells in culture may enhance apolipoprotein-mediated lipid release. Treatment of fibroblasts with interleukin-1β has been shown [22] to increase efflux to apolipoprotein AI. An increase of intracellular free cholesterol increases both cholesterol and phospholipid efflux to apolipoproteins. Furthermore, cyclic AMP may also enhance specific binding of different apoproteins and transfer of lipids to the membrane. Cyclic AMP appears to impact on cholesterol efflux by increasing cholesterol ester hydrolysis, as well as the movement of free cholesterol from intracellular sites to the plasma membrane [23]. Interestingly, defects in this process have been reported in patients with Tangier disease [24]. In mouse macrophage cell lines, cyclic AMP causes a dramatic increase in internalization of apolipoprotein AI, followed by its resecretion in association with cholesterol, providing insights as to how apolipoprotein AI may stimulate efflux [25•].

Probucol has long been known to lower HDL-cholesterol levels, and one mechanism for this has now been shown to be reduced apolipoprotein AI-mediated cholesterol efflux. The effect of probucol on efflux appears to be generalized and not limited to specific cells. By *contrast*, a positive influence of cyclic AMP on cholesterol efflux appears to be cell-type specific and is only seen, for example, in certain macrophage cell lines, and not in human fibroblasts. This also suggests that some, but not all, influences on cholesterol efflux are cell-specific [23].

SR-BI is a scavenger receptor that binds HDL with high affinity and mediates selective uptake of cholesteryl ester from HDL. Recently, it has also been demonstrated that SR-BI can stimulate free cholesterol flux from Chinese hamster ovary cells that express mouse SR-BI, demonstrating that SR-BI can mediate alterations to membrane cholesterol traffic, provoking bidirectional cholesterol flux [26,27].

Cholesterol efflux regulatory protein, Tangier disease, and HDL deficiency

In humans, defects in cholesterol efflux are most evident in patients with Tangier disease, which manifests with marked deficiency of HDL and cholesterol ester accumulation in specific cells. Furthermore, depressed levels of cholesterol efflux are also seen in some patients with familial HDL deficiency (FHA). Recently, mutations in the *ABC1* gene were found to underlie both Tangier disease and this form of FHA.

The gene for Tangier disease was mapped to 9q31 towards the end of 1998 [28]. Thereafter, different approaches were taken in different laboratories to clone

the Tangier disease gene. These included analysis of expression of genes moderated by cholesterol accumulation in cells [11••,12••], followed by mapping these to specific regions of the human genome, as well as by classical positional cloning strategies [10••].

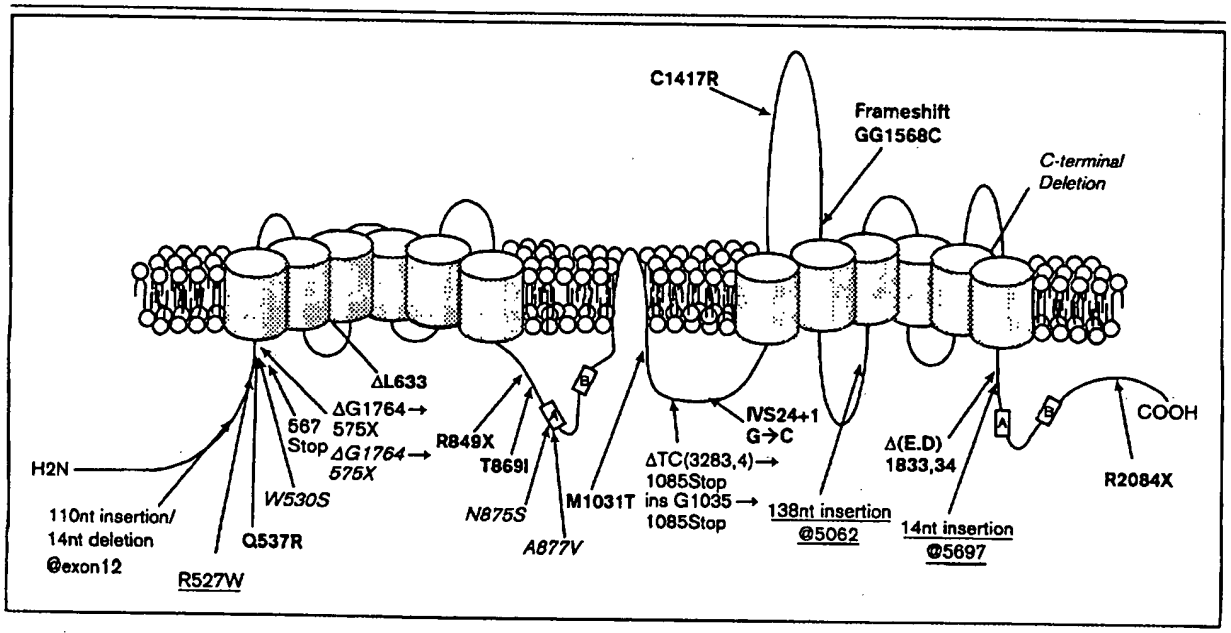
We undertook two separate classical positional cloning approaches to identify the genes that underlie Tangier disease and FHA [10••].To that point, it had been recognized that FHA had features of an autosomal dominant disorder characterized by clear manifestations of low HDL in the heterozygote, with no obvious features of cholesterol ester deposition in cells [29]. By contrast, Tangier disease had been defined as an autosomal recessive illness with clear evidence for cholesterol deposition in cells and less obvious manifestations in the heterozygote. Furthermore, even though both diseases had some evidence for cholesterol efflux, this did not necessarily mean that this was due to defects in the same gene. Numerous genetic disorders with common phenotypic features may be caused by defects in different genes (e.g. genes affecting hypertension). In addition, in different diseases, defects in multiple genes may be associated with a very similar phenotype (for example diabetes and Hirschsprung's disease).

Genetic analysis revealed that the gene for FHA mapped to the same region as the mutation that underlies Tangier disease [10••].This suggested that Tangier disease and FHA were due to mutations in the same gene, or potentially these mutations were due to different genes in close proximity to each other. Further analysis, however, revealed that these diseases were allelic with Tangier disease, resulting from mutations on both alleles of *ABC1*, whereas FHA was due to a mutation on a single allele.

ABC1 is a member of the ATP-binding cassette family of genes, a large and rapidly expanding gene family currently containing about 50 members. Although all *ABC* family members contain an ATP-binding cassette that consists of consensus Walker A and Walker B motifs, as well as membrane-spanning sequences, they vary widely in the arrangement of their protein domains. Some, referred to as 'half-transporters', contain only one ATP-binding site and a transmembrane domain consisting of six membrane-spanning segments. *ABC1* itself contains two ATP-binding cassettes, two membrane-spanning domains with six transmembrane domains each, a large extracellular loop and a long amino-terminal segment (Fig. 1).

Heterozygotes for Tangier disease are genetically similar to those of patients with FHA. Why, then, was Tangier disease regarded as inherited as a recessive trait, whereas FHA was presumed to be dominant? Analysis of the

Figure 1. Schematic diagram of the cholesterol efflux regulatory protein



Schematic diagram of the cholesterol efflux regulatory protein, showing the location of all mutations reported to date ([12••] – italics; [11••] – light grey; [10••,30•] – black; [31••] – underlined). The critical ATP-binding cassette region is characterized by the Walker A and B motifs, designated A and B, respectively.

families [10••,30•] provides some explanation. Dominance implies that the phenotype is manifest in the heterozygote. Our first families assessed for FHA in general had more severe mutations that were associated more frequently with HDL levels below 5% for age and sex (Fig. 1). These low HDL levels increased the likelihood of ascertainment. Mutations that result in truncation of the protein or major deletions are more often seen in persons with HDL below 5%. By contrast, the two families with Tangier disease that we studied [10••] had missense mutations and, in all likelihood, these mutations may give rise to proteins with significant functional deficits, but retaining some residual activity (Fig. 2).

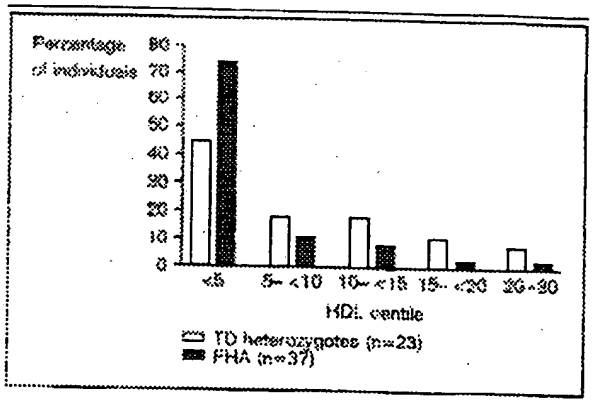
The *ABCR* gene, which encodes a retinal rod photoreceptor protein, has the strongest homology to CERP (approximately 50%) of any ABC protein. Interestingly, persons who are heterozygous for mutations in *ABCR* have a phenotype (e.g. adult-onset muscular dystrophy), which was not previously thought to be related to the phenotype of retinitis pigmentosa that is due to mutations on both alleles [32]. For mutations in *ABCR* and *ABC1*, the clinical spectrum is likely to vary dependent on the functional effects on the protein. Thus, we would predict a broader clinical spectrum for patients with mutations on both alleles, particularly when one of these alleles results in an *ABC1* protein with some residual activity.

The distribution of Tangier disease and FHA mutations in the *ABC1* gene can provide information about the role of CERP and its different domains. Many of the missense mutations seen in Tangier disease or FHA families occur in or near the ATP-binding cassettes, with fewer mutations in the membrane-spanning segments (Fig. 1). This is consistent with active cholesterol efflux being an energy-requiring process that is dependent on ATP hydrolysis.

Another important question is whether different sites of mutation are more likely to be associated with a particular phenotype, in particular coronary artery disease. Preliminary assessment has revealed that the likelihood of coronary disease does not correlate with the location of the mutation. Rather, it is likely to be reflective of the remaining function of CERP, in addition to other genetic and environmental factors.

Mutations in CERP are associated with significant defects in cholesterol efflux. The fact that patients with Tangier disease have such marked reduction in efflux demonstrates the importance of CERP *in vivo* in the efflux of cholesterol or phospholipid from cells, and the importance of this first step in the regulation

Figure 2. HDL-cholesterol levels



HDL-cholesterol levels in heterozygous carriers of cholesterol efflux regulatory protein (CERP) mutations from Tangier disease (TD) and familial HDL deficiency (FHA) families are shown. The percentage of individuals from TD or FHA families with HDL-cholesterol levels falling in a given range of centiles for age and sex are plotted. Individuals from the FHA families (*n*=37) are more often found with HDL in the lower centile categories than those from the TD families (*n*=23).

of HDL levels in humans. Clearly, lipidation of nascent HDL represents a critical step in the maintenance and regulation of HDL levels in humans (Fig. 3).

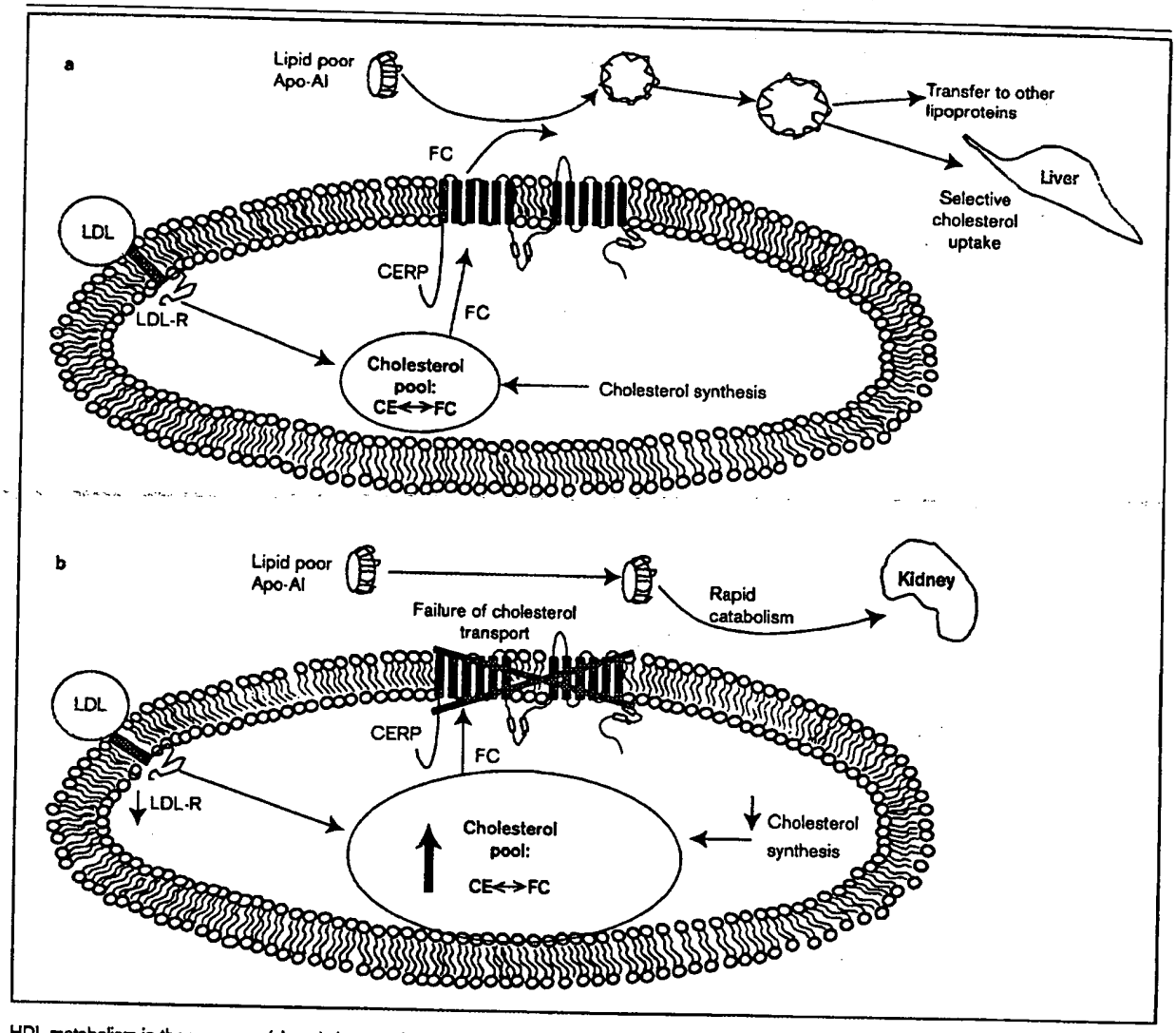
An important question is whether defects in other proteins are likely to be contributing to this major deficit in efflux. Clearly, mutations in other proteins can be associated with defects in cholesterol efflux, such as mutations in particular domains of apolipoprotein AI. Analysis of the majority of the patients and families who presented with cholesterol efflux defects, however, has revealed that mutations in CERP represent the major but not the only determinant for cholesterol efflux in humans.

Determination of HDL levels is multigenic and it is not surprising that the phenotype in heterozygotes for CERP deficiency varies not only on the basis of the nature of the mutation, but also on the basis of other factors that may influence HDL levels. For example, age appears to partly influence the manifestation of the phenotype, with more variability of the phenotype seen in patients less than 20 years of age (data not shown).

Conclusion

Discovery of the effect of CERP in the determination of HDL levels in humans confirms the role of RCT in the maintenance of HDL levels in humans. Although CERP is a major determinant of efflux, clearly other as yet

Figure 3. HDL metabolism



HDL metabolism in the presence (a) and absence (b) of cholesterol efflux regulatory protein (CERP) function. When CERP is present and cholesterol is efficiently mobilized from intracellular stores and transferred to lipid-poor apolipoprotein AI (Apo-AI), forming mature HDL particles, which can then function in the reverse cholesterol transport process. In the absence of CERP function, cholesterol ester accumulates within the cell, and the lipid-poor Apo-AI particles are not lipidated and are rapidly catabolized. CE, cholesterol ester; FC, free cholesterol; LDL-R, LDL receptor.

undiscovered proteins are likely to influence this process. Identification of CERP has provided a critical platform from which we may now begin to elucidate the functioning of the RCT pathway, and the regulation of HDL-cholesterol levels in humans.

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- •• of outstanding interest

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1: AJ012376. Homo sapiens mRNA...[gi:4128032] Links

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  TITLE     Molecular Cloning of the Human ATP-Binding Cassette Transporter 1
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DT   15-JUN-2002 (Rel. 41, Last annotation update)
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RT   "Cloning of two novel ABC transporters mapping on human chromosome
RT   9.";
RL   Genomics 21:150-159(1994).
RN   [2]
RP   SEQUENCE FROM N.A.
RC   STRAIN=C57BL/6J;
RA   Qiu Y., Cavelier L., Chiu S., Rubin E., Cheng J.-F.;
RT   "Human and mouse ABCA1 comparative sequencing and transgenesis studies
RT   identify potential regulatory sequences.";
RL   Submitted (JUL-2000) to the EMBL/GenBank/DDBJ databases.
CC   -!- FUNCTION: CAMP-DEPENDENT AND SULFONYLUREA-SENSITIVE ANION
CC       TRANSPORTER. KEY GATEKEEPER INFLUENCING INTRACELLULAR CHOLESTEROL
CC       TRANSPORT (BY SIMILARITY).
CC   -!- TISSUE SPECIFICITY: WIDELY EXPRESSED IN ADULT TISSUES. HIGHEST
CC       LEVELS ARE FOUND IN PREGNANT UTERUS AND UTERUS.
CC   -!- DOMAIN: MULTIFUNCTIONAL POLYPEPTIDE WITH TWO HOMOLOGOUS HALVES,
CC       EACH CONTAINING AN HYDROPHOBIC MEMBRANE-ANCHORING DOMAIN AND AN
CC       ATP BINDING CASSETTE (ABC) DOMAIN.
CC   -!- SIMILARITY: BELONGS TO THE ABC TRANSPORTER FAMILY. ABCA SUBFAMILY.
CC   ----------------------------------------------------------------------
CC   This SWISS-PROT entry is copyright. It is produced through a collaboration
CC   between the Swiss Institute of Bioinformatics and the EMBL outstation -
CC   the European Bioinformatics Institute. There are no restrictions on its
CC   use by non-profit institutions as long as its content is in no way
CC   modified and this statement is not removed. Usage by and for commercial
CC   entities requires a license agreement (See http://www.isb-sib.ch/announce/
CC   or send an email to license@isb-sib.ch).
CC   ----------------------------------------------------------------------
DR   EMBL; X75926; CAA53530.1; ALT_INIT.
DR   EMBL; AF287263; AAG39073.1; ALT_INIT.
DR   MGD; MGI:99607; Abca1.
DR   InterPro; IPR003593; AAA_ATPase.
DR   InterPro; IPR003439; ABC_transportr.
DR   Pfam; PF00005; ABC_tran; 2.
DR   ProDom; PD000006; ABC_transportr; 2
DR   SMART; SM00382; AAA; 2.
DR   PROSITE; PS00211; ABC_TRANSPORTER; 1.
KW   ATP-binding; Glycoprotein; Transmembrane; Transport.
FT   TRANSMEM     26     42       POTENTIAL.
FT   TRANSMEM    640    656       POTENTIAL.
FT   TRANSMEM    690    706       POTENTIAL.
FT   TRANSMEM    717    733       POTENTIAL.
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FT   TRANSMEM    749    765       POTENTIAL.
FT   TRANSMEM    771    787       POTENTIAL.
FT   TRANSMEM   1041   1057       POTENTIAL.
FT   TRANSMEM   1351   1367       POTENTIAL.
FT   TRANSMEM   1661   1677       POTENTIAL.
FT   TRANSMEM   1708   1724       POTENTIAL.
FT   TRANSMEM   1737   1753       POTENTIAL.
FT   TRANSMEM   1775   1791       POTENTIAL.
FT   TRANSMEM   1854   1870       POTENTIAL.
FT   NP_BIND     933    940       ATP (POTENTIAL).
FT   NP_BIND    1946   1953       ATP (POTENTIAL).
FT   CARBOHYD     14     14       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD     98     98       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    151    151       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    161    161       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    196    196       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    244    244       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    292    292       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    337    337       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    349    349       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    400    400       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    478    478       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    489    489       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    521    521       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD    820    820       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD   1144   1144       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD   1294   1294       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD   1453   1453       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD   1499   1499       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD   1504   1504       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD   1637   1637       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD   2044   2044       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CARBOHYD   2238   2238       N-LINKED (GLCNAC. . .) (POTENTIAL).
FT   CONFLICT   1567   1568       MISSING (IN REF. 2).
FT   CONFLICT   2024   2024       MISSING (IN REF. 2).
SQ   SEQUENCE   2261 AA;  254011 MW;  FAE62B21FD1D09F9 CRC64;

  Query Match             95.4%;  Score 11256;  DB 1;  Length 2261;
  Best Local Similarity   95.0%;  Pred. No. 0;
  Matches 2149;  Conservative   55;  Mismatches   57;  Indels    0;  Gaps    0;

Qy       1 MACWPQLRLLLWKNLTFRRRQTCQLLLEVAWPLFIFLILISVRLSYPPYEQHECHFPNKA 60
           |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Db       1 MACWPQLRLLLWKNLTFRRRQTCQLLLEVAWPLFIFLILISVRLSYPPYEQHECHFPNKA 60

Qy      61 MPSAGTLPWVQGIICNANNPCFRYPTPGEAPGVVGNFNKSIVARLFSDARRLLLYSQKDT 120
           ||||||||||||||||||||||||||||||||||||||||||||:||||||:|||||||:||
Db      61 MPSAGTLPWVQGIICNANNPCFRYPTPGEAPGVVGNFNKSIVSRLFSDAQRLLLYSQRDT 120

Qy     121 SMKDMRKVLRTLQQIKKSSSNLKLQDFLVDNETFSGFLYRNLSLPKSTVDKMLRADVILH 180
           |:||| |||| |:||  :|||||||||||||||| |||||:|||| :|: :| | |
Db     121 SIKDMHKVLRMLRQIKHPNSNLKLQDFLVDNETFSGFLQHNDSLPRSTVDSLLQXNVGLQ 180

Qy     181 KVFLQGYQLHLTSLCNGSKSEEMIQLGDQEVSELCGLPKEKLAAAERVLRSNMDILKPIL 240
           |||||||||| ||||||| ||:||||| ||| |||||::|| |||||| |||||||::
Db     181 KVFLQGYQLHLASLCNGSKLEEIIQLGDAEVSALCGLPRKKLDAAERVLRYNMDILKPVV 240

Qy     241 RTLNSTSPFPSKELAEATKTLLHSLGTLAQELFSMRSWSDMRQEVMFITNVNSSSSSTQI 300
           |||||  |:: |||||  || ||| ||||||| :|||||||||||||||||||||||||||
Db     241 TKLNSTSHLPTQHLAEATTVLLDSLGGLAQELFSTKSWSDMRQEVMFLTNVNSSSSSTQI 300

Qy     301 YQAVSRIVCGHPEGGGLKIKSLNWYEDNNYKALFGGNGTEEDAETFYDNSTTPYCNDLMK 360
           |||||||||||||||||||||||||||||||||||||||||| |||| :|||||||||||||
Db     301 YQAVSRIVCGHPEGGGLKIKSLNWYEDNNYKALFGGNNTEEDVDTFYDNSTTPYCNDLMK 360

Qy     361 NLESSPLSRITWKALKPLLVGKILYTPDTPATRQVMAEVNKTFQELAVFHDLEGMWEELS 420
           ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Db     361 NLESSPLSRITWKALKPLLVGKILYTPDTPATRQVMAEVNKTFQELAVFHDLEGMWEELS 420

Qy     421 PKIWTFMENSQEMDLVRMLLDSRDNDHFWEQQLDGLDWTAQDIVAFLAKHPEDVQSSNGS 480
           |:||||||||||||||| ||||| || ||||:||||||||||:||||:|||||| |||
Db     421 PQIWTFMENSQEMDLVRTLLDSRGNDQFWEQKLDGLDWTAQDIMAFLAKNPEDVQSPNGS 480
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PATENT

UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. 99,395-A)

IN THE APPLICATION OF:			
Lawn et al.			
Serial No.	09/595,526	Examiner:	Rao, Manjunath N.
Filed:	June 16, 2000	Group Art Unit:	1652
Title:	Compositions and Methods For Increasing Cholesterol Efflux and Raising HDL, Using ATP Binding Cassette Transporter Protein ABC1	Confirmation No.:	9969

DECLARATION OF RICHARD LAWN, PH.D. UNDER 37 C.F.R. §1.131

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Richard Lawn, Ph.D., declare as follows:

(1) I am a named inventor of the above-mentioned U.S. Patent Application Serial Number 09/595,526.

(2) The inventions described in the claims of Patent Application Serial Number 09/595,526 were conceived and reduced to practice cooperatively by the named inventors, Dr. Richard Lawn, Dr. David Wade, Dr. Michael Garvin, and Dr. John Oran, prior to May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.

(3) The amino acid sequence of ABC1 protein, which appears as SEQ ID NO: 2 in the above-mentioned application, and the corresponding nucleotide sequence of the open reading frame of the ABC1 gene (cDNA), which appears as nucleotides 291-7074 of SEQ ID NO: 1 in the above-mentioned

application, were reduced to practice before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.

(4) The nucleotide sequence of ABC1 gene (cDNA) including the 5' and 3' untranslated sequences, which appears as SEQ ID NO: 1 in the above-mentioned application, was reduced to practice before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.

(5) Attached as Exhibit A is a copy of two laboratory notebook pages (from which the dates have been redacted) that demonstrate that the cDNA clone of the ABC1 gene was isolated before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.

(6) Attached as Exhibit B is a copy of the 4-page sequence report (from which the dates have been redacted) that demonstrates that the nucleotide sequence of ABC1 gene (cDNA), which appears as SEQ ID NO: 1 in the above-mentioned application was sequenced before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.

(7) Attached as Exhibit C is a copy of four laboratory notebook pages (from which the dates have been redacted) that demonstrate that the extended open reading frame of ABC-1 gene (cDNA; nucleotides 291-7074 of SEQ ID NO: 1) was isolated before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.

(8) Attached as Exhibit D is a copy of a one-page sequence report (from which the dates have been redacted) that demonstrates that the amino acid sequence of ABC1 protein (predicted from translation of the nucleotide sequence) which appears as SEQ ID NO: 2 in the above-mentioned application was sequenced before May 2, 2000, the priority date for Rosier-Montus et al. US 20020146792A1.

(9) I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge

imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Respectfully submitted,

Date: Sept 25, 2003

By: Richard M Lawn

Richard Lawn, Ph.D.

From Page No.

Selecting ABC1 from pCEP4 Library using RecA kit

1. Isolate total library DNA using Qiagen kit.
2. ~~Lab~~ Label 600 bp PCR product from 5' end of ABC1 using biotin.
3. Select according to Clontech instructions.
4. Transform 1 µl of plasmid DNA into DH10B electrocompetent cells.
   Got ~ 7000 colonies total.
5. Isolate plasmid from all the transformants after O.N. growth in amp.
6. Carry another round of ~~selecting~~ selection under the same conditions as round one, and transform DH10B w/ 1 µl of DNA
   Got ~ 150,000 colonies total.
7. Spread 1000 cells on one 150 mm agar plate from round one,
   and 500 cells " " two.
8. Do colony lift on the plates and hybridize to the 600 bp probe.
   found 0 positives on round 1 filter, 3 positives on round 2 filter.
   the 3rd colony was too close to another colony. so picked the 4th colony in case of misalignment.

To Page No.

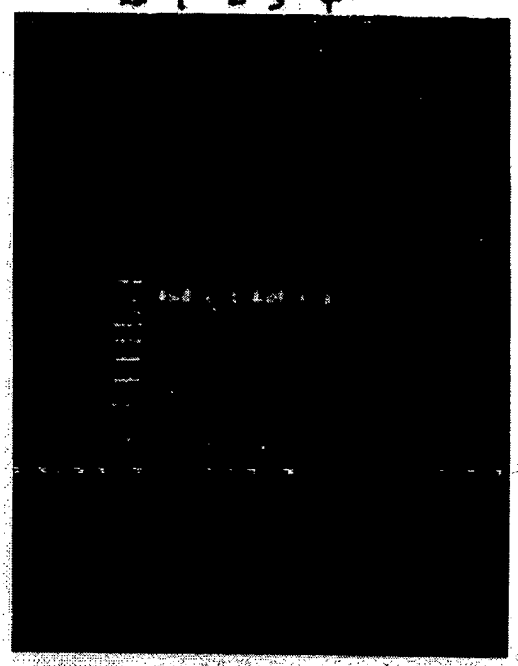
Witnessed & Understood by me.	Date	Invented by Xinghua	Date
		Recorded by Xinghua	

From Page No. ___

9. prepare miniprep plasmid DNA and cut w/ Hind III & Xho I (cloning sites)

colony 1 showed two fragments of 5.5 kb & 2.9 kb

colony 2 & 3 showed very weak ~1kb bands, but not captured by the photograph



10. PCR to confirm the existance of the probe sequence in the picked clones.

Using the primers at the ends of the 600 bp probe.

Template:

Lane P. 10 ng of probe

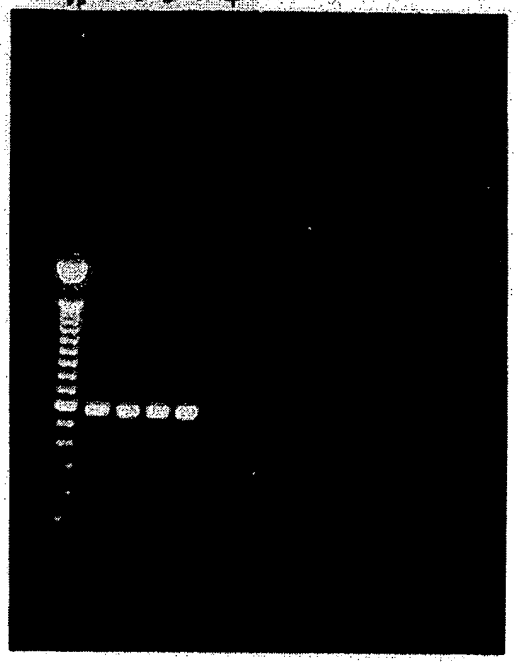
1. 1 µl of clone 1 miniprep

2. " 2

3 3

4 4

clone 4 whowed no band at all, indicating the absence of PCR contamination.



To Page No. ___

Witnessed & Understood by me,	Date /	Invented by Xinli Wang	Date
		Recorded by Xinli Wang	

CVThABC1 NUCLEOTIDE 10442 BP
Start codon: 291
Stop codon :7074
Genbank CDS : 350 to 7230
Overlap with genomic fragment pAPR1 5' promoter: 1 to 67

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   1 GGCCGGGACC CGCAGAGCCG AGCCGACCCT TCTCTCCCGG GCTGCGGCAG GGCAGGGCGG
  61 GGAGCTCCGC GCACCAACAG AGCCGGTTCT CAGGGCGCTT TGCTCCTTGT TTTTTCCCCG
 121 GTTCTGTTTT CTCCCCTTCT CCGGAAGGCT TGTCAAGGGG TAGGAGAAAG AGACGCAAAC
 181 ACAAAAGTGG AAAACAGTTA ATGACCAGCC ACGGGCGTCC CTGCTGTGAG CTCTGGCCGC
 241 TGCCTTCCAG GGCTCCCGAG CCACACGCTG GGCGTGCTGG CTGAGGGAAC ATGGCTTGTT
 301 GGCCTCAGCT GAGGTTGCTG CTGTGGAAGA ACCTCACTTT CAGAAGAAGA CAAACATGTC
 361 AGCTGTTACT GGAAGTGGCC TGGCCTCTAT TTATCTTCCT GATCCTGATC TCTGTTCGGC
 421 TGAGCTACCC ACCCTATGAA CAACATGAAT GCCATTTTCC AAATAAAGCC ATGCCCTCTG
 481 CAGGAACACT TCCTTGGGTT CAGGGGATTA TCTGTAATGC CAACAACCCC TGTTTCCGTT
 541 ACCCGACTCC TGGGGAGGCT CCCGGAGTTG TTGGAAACTT TAACAAATCC ATTGTGGCTC
 601 GCCTGTTCTC AGATGCTCGG AGGCTTCTTT TATACAGCCA GAAAGACACC AGCATGAAGG
 661 ACATGCGCAA AGTTCTGAGA ACATTACAGC AGATCAAGAA ATCCAGCTCA AACTTGAAGC
 721 TTCAAGATTT CCTGGTGGAC AATGAAACCT TCTCTGGGTT CCTATATCAC AACCTCTCTC
 781 TCCCAAAGTC TACTGTGGAC AAGATGCTGA GGGCTGATGT CATTCTCCAC AAGGTATTTT
 841 TGCAAGGCTA CCAGTTACAT TTGACAAGTC TGTGCAATGG ATCAAAATCA GAAGAGATGA
 901 TTCAACTTGG TGACCAAGAA GTTTCTGAGC TTTGTGGCCT ACCAAAGGAG AAACTGGCTG
 961 CAGCAGAGCG AGTACTTCGT TCCAACATGG ACATCCTGAA GCCAATCCTG AGAACACTAA
1021 ACTCTACATC TCCCTTCCCG AGCAAGGAGC TGGCTGAAGC CACAAAAACA TTGCTGCATA
1081 GTCTTGGGAC TCTGGCCCAG GAGCTGTTCA GCATGAGAAG CTGGAGTGAC ATGCGACAGG
1141 AGGTGATGTT TCTGACCAAT GTGAACAGCT CCAGCTCCTC CACCCAAATC TACCAGGCTG
1201 TGTCTCGTAT TGTCTGCGGG CATCCCGAGG GAGGGGGGCT GAAGATCAAG TCTCTCAACT
1261 GGTATGAGGA CAACAACTAC AAAGCCCTCT TTGGAGGCAA TGGCACTGAG GAAGATGCTG
1321 AAACCTTCTA TGACAACTCT ACAACTCCTT ACTGCAATGA TTTGATGAAG AATTTGGAGT
1381 CTAGTCCTCT TTCCCGCATT ATCTGGAAAG CTCTGAAGCC GCTGCTCGTT GGGAAGATCC
1441 TGTATACACC TGACACTCCA GCCACAAGGC AGGTCATGGC TGAGGTGAAC AAGACCTTCC
1501 AGGAACTGGC TGTGTTCCAT GATCTGGAAG GCATGTGGGA GGAACTCAGC CCCAAGATCT
1561 GGACCTTCAT GGAGAACAGC CAAGAAATGG ACCTTGTCCG GATGCTGTTG GACAGCAGGG
1621 ACAATGACCA CTTTTGGGAA CAGCAGTTGG ATGGCTTAGA TTGGACAGCC CAAGACATCG
1681 TGGCGTTTTT GGCCAAGCAC CCAGAGGATG TCCAGTCCAG TAATGGTTCT GTGTACACCT
1741 GGAGAGAAGC TTTCAACGAG ACTAACCAGG CAATCCGGAC CATATCTCGC TTCATGGAGT
1801 GTGTCAACCT GAACAAGCTA GAACCCATAG CAACAGAAGT CTGGCTCATC AACAAGTCCA
1861 TGGAGCTGCT GGATGAGAGG AAGTTCTGGG CTGGTATTGT GTTCACTGGA ATTACTCCAG
1921 GCAGCATTGA GCTGCCCCAT CATGTCAAGT ACAAGATCCG AATGGACATT GACAATGTGG
1981 AGAGGACAAA TAAAATCAAG GATGGGTACT GGGACCCTGG TCCTCGAGCT GACCCCTTTG
2041 AGGACATGCG GTACGTCTGG GGGGGCTTCG CCTACTTGCA GGATGTGGTG GAGCAGGCAA
2101 TCATCAGGGT GCTGACGGGC ACCGAGAAGA AAACTGGTGT CTATATGCAA CAGATGCCCT
2161 ATCCCTGTTA CGTTGATGAC ATCTTTCTGC GGGTGATGAG CCGGTCAATG CCCCTCTTCA
2221 TGACGCTGGC CTGGATTTAC TCAGTGGCTG TGATCATCAA GGGCATCGTG TATGAGAAGG
2281 AGGCACGGCT GAAAGAGACC ATGCGGATCA TGGGCCTGGA CAACAGCATA CTCTGGTTTA
2341 GCTGGTTCAT TAGTAGCCTC ATTCCTCTTC TTGTGAGCGC TGGCCTGCTA GTGGTCATCC
2401 TGAAGTTAGG AAACCTGCTG CCCTACAGTG ATCCCAGCGT GGTGTTTGTC TTCCTGTCCG
2461 TGTTTGCTGT GGTGACAATC CTGCAGTGCT TCCTGATTAG CACACTCTTC TCCAGAGCCA
2521 ACCTGGCAGC AGCCTGTGGG GGCATCATCT ACTTCACGCT GTACCTGCCC TACGTCCTGT
2581 GTGTGGCATG GCAGGACTAC GTGGGCTTCA CACTCAAGAT CTTCGCTAGC CTGCTGTCTC
2641 CTGTGGCTTT TGGGTTTGGC TGTGAGTACT TTGCCCTTTT TGAGGAGCAG GGCATTGGAG
2701 TGCAGTGGGA CAACCTGTTT GAGAGTCCTG TGGAGGAAGA TGGCTTCAAT CTCACCACTT
2761 CGATCTCCAT GATGCTGTTT GACACCTTCC TCTATGGGGT GATGACCTGG TACATTGAGG
2821 CTGTCTTTCC AGGCCAGTAC GGAATTCCCA GGCCCTGGTA TTTTCCTTGC ACCAAGTCCT
2881 ACTGGTTTGG CGAGGAAAGT GATGAGAAGA GCCACCCTGG TTCCAACCAG AAGAGAATGT
2941 CAGAAATCTG CATGGAGGAG GAACCCACCC ACTTGAAGCT GGGCGTGTCC ATTCAGAACC
3001 TGGTAAAAGT CTACCGAGAT GGGATGAAGG TGGCTGTCGA TGGCCTGGCA CTGAATTTTT
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3061 ATGAGGGCCA GATCACCTCC TTCCTGGGCC ACAATGGAGC GGGGAAGACG ACCACCATGT
3121 CAATCCTGAC CGGGTTGTTC CCCCCGACCT CGGGCACCGC CTACATCCTG GGAAAAGACA
3181 TTCGCTCTGA GATGAGCACC ATCCGGCAGA ACCTGGGGGT CTGTCCCCAG CATAACGTGC
3241 TGTTTGACAT GCTGACTGTC GAAGAACACA TCTGGTTCTA TGCCCGCTTG AAAGGGCTCT
3301 CTGAGAAGCA CGTGAAGGCG GAGATGGAGC AGATGGCCCT GGATGTTGGT TTGCCATCAA
3361 GCAAGCTGAA AAGCAAAACA AGCCAGCTGT CAGGTGGAAT GCAGAGAAAG CTATCTGTGG
3421 CCTTGGCCTT TGTCGGGGGA TCTAAGGTTG TCATTCTGGA TGAACCCACA GCTGGTGTGG
3481 ACCCTTACTC CCGCAGGGGA ATATGGGAGC TGCTGCTGAA ATACCGACAA GGCCGCACCA
3541 TTATTCTCTC TACACACCAC ATGGATGAAG CGGACGTCCT GGGGGACAGG ATTGCCATCA
3601 TCTCCCATGG GAAGCTGTGC TGTGTGGGCT CCTCCCTGTT TCTGAAGAAC CAGCTGGGAA
3661 CAGGCTACTA CCTGACCTTG GTCAAGAAAG ATGTGGAATC CTCCCTCAGT TCCTGCAGAA
3721 ACAGTAGTAG CACTGTGTCA TACCTGAAAA AGGAGGACAG TGTTTCTCAG AGCAGTTCTG
3781 ATGCTGGCCT GGGCAGCGAC CATGAGAGTG ACACGCTGAC CATCGATGTC TCTGCTATCT
3841 CCAACCTCAT CAGGAAGCAT GTGTCTGAAG CCCGGCTGGT GGAAGACATA GGGCATGAGC
3901 TGACCTATGT GCTGCCATAT GAAGCTGCTA AGGAGGGAGC CTTTGTGGAA CTCTTTCATG
3961 AGATTGATGA CCGGCTCTCA GACCTGGGCA TTTCTAGTTA TGGCATCTCA GAGACGACCC
4021 TGGAAGAAAT ATTCCTCAAG GTGGCCGAAG AGAGTGGGGT GGATGCTGAG ACCTCAGATG
4081 GTACCTTGCC AGCAAGACGA AACAGGCGGG CCTTCGGGGA CAAGCAGAGC TGTCTTCGCC
4141 CGTTCACTGA AGATGATGCT GCTGATCCAA ATGATTCTGA CATAGACCCA GAATCCAGAG
4201 AGACAGACTT GCTCAGTGGG ATGGATGGCA AAGGGTCCTA CCAGGTGAAA GGCTGGAAAC
4261 TTACACAGCA ACAGTTTGTG GCCCTTTTGT GGAAGAGACT GCTAATTGCC AGACGGAGTC
4321 GGAAAGGATT TTTTGCTCAG ATTGTCTTGC CAGCTGTGTT TGTCTGCATT GCCCTTGTGT
4381 TCAGCCTGAT CGTGCCACCC TTTGGCAAGT ACCCCAGCCT GGAACTTCAG CCCTGGATGT
4441 ACAACGAACA GTACACATTT GTCAGCAATG ATGCTCCTGA GGACACGGGA ACCCTGGAAC
4501 TCTTAAACGC CCTCACCAAA GACCCTGGCT TCGGGACCCG CTGTATGGAA GGAAACCCAA
4561 TCCCAGACAC GCCCTGCCAG GCAGGGGAGG AAGAGTGGAC CACTGCCCCA GTTCCCCAGA
4621 CCATCATGGA CCTCTTCCAG AATGGGAACT GGACAATGCA GAACCCTTCA CCTGCATGCC
4681 AGTGTAGCAG CGACAAAATC AAGAAGATGC TGCCTGTGTG TCCCCCAGGG GCAGGGGGGC
4741 TGCCTCCTCC ACAAAGAAAA CAAAACACTG CAGATATCCT TCAGGACCTG ACAGGAAGAA
4801 ACATTTCGGA TTATCTGGTG AAGACGTATG TGCAGATCAT AGCCAAAAGC TTAAAGAACA
4861 AGATCTGGGT GAATGAGTTT AGGTATGGCG GCTTTTCCCT GGGTGTCAGT AATACTCAAG
4921 CACTTCCTCC GAGTCAAGAA GTTAATGATG CCATCAAACA AATGAAGAAA CACCTAAAGC
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5041 TGGACACCAG AAATAATGTC AAGGTGTGGT TCAATAACAA GGGCTGGCAT GCAATCAGCT
5101 CTTTCCTGAA TGTCATCAAC AATGCCATTC TCCGGGCCAA CCTGCAAAAG GGAGAGAACC
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5221 CAGAGGTGGC TCTGATGACC ACATCAGTGG ATGTCCTTGT GTCCATCTGT GTCATCTTTG
5281 CAATGTCCTT CGTCCCAGCC AGCTTTGTCG TATTCCTGAT CCAGGAGCGG GTCAGCAAAG
5341 CAAAACACCT GCAGTTCATC AGTGGAGTGA AGCCTGTCAT CTACTGGCTC TCTAATTTTG
5401 TCTGGGATAT GTGCAATTAC GTTGTCCCTG CCACACTGGT CATTATCATC TTCATCTGCT
5461 TCCAGCAGAA GTCCTATGTG TCCTCCACCA ATCTGCCTGT GCTAGCCCTT CTACTTTTGC
5521 TGTATGGGTG GTCAATCACA CCTCTCATGT ACCCAGCCTC CTTTGTGTTC AAGATCCCCA
5581 GCACAGCCTA TGTGGTGCTC ACCAGCGTGA ACCTCTTCAT TGGCATTAAT GGCAGCGTGG
5641 CCACCTTTGT GCTGGAGCTG TTCACCGACA ATAAGCTGAA TAATATCAAT GATATCCTGA
5701 AGTCCGTGTT CTTGATCTTC CCACATTTTT GCCTGGGACG AGGGCTCATC GACATGGTGA
5761 AAAACCAGGC AATGGCTGAT GCCCTGGAAA GGTTTGGGGA GAATCGCTTT GTGTCACCAT
5821 TATCTTGGGA CTTGGTGGGA CGAAACCTCT TCGCCATGGC CGTGGAAGGG GTGGTGTTCT
5881 TCCTCATTAC TGTTCTGATC CAGTACAGAT TCTTCATCAG GCCCAGACCT GTAAATGCAA
5941 AGCTATCTCC TCTGAATGAT GAAGATGAAG ATGTGAGGCG GGAAAGACAG AGAATTCTTG
6001 ATGGTGGAGG CCAGAATGAC ATCTTAGAAA TCAAGGAGTT GACGAAGATA TATAGAAGGA
6061 AGCGGAAGCC TGCTGTTGAC AGGATTTGCG TGGGCATTCC TCCTGGTGAG TGCTTTGGGC
6121 TCCTGGGAGT TAATGGGGCT GGAAAATCAT CAACTTTCAA GATGTTAACA GGAGATACCA
6181 CTGTTACCAG AGGAGATGCT TTCCTTAACA AAAATAGTAT CTTATCAAAC ATCCATGAAG
6241 TACATCAGAA CATGGGCTAC TGCCCTCAGT TTGATGCCAT CACAGAGCTG TTGACTGGGA
6301 GAGAACACGT GGAGTTCTTT GCCCTTTTGA GAGGAGTCCC AGAGAAAGAA GTTGGCAAGG
6361 TTGGTGAGTG GGCGATTCGG AAACTGGGCC TCGTGAAGTA TGGAGAAAAA TATGCTGGTA
6421 ACTATAGTGG AGGCAACAAA CGCAAGCTCT CTACAGCCAT GGCTTTGATC GGCGGGCCTC
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6481 CTGTGGTGTT TCTGGATGAA CCCACCACAG GCATGGATCC CAAAGCCCGG CGGTTCTTGT
6541 GGAATTGTGC CCTAAGTGTT GTCAAGGAGG GGAGATCAGT AGTGCTTACA TCTCATAGTA
6601 TGGAAGAATG TGAAGCTCTT TGCACTAGGA TGGCAATCAT GGTCAATGGA AGGTTCAGGT
6661 GCCTTGGCAG TGTCCAGCAT CTAAAAAATA GGTTTGGAGA TGGTTATACA ATAGTTGTAC
6721 GAATAGCAGG GTCCAACCCG GACCTGAAGC CTGTCCAGGA TTTCTTTGGA CTTGCATTTC
6781 CTGGAAGTGT TCTAAAAGAG AAACACCGGA ACATGCTACA ATACCAGCTT CCATCTTCAT
6841 TATCTTCTCT GGCCAGGATA TTCAGCATCC TCTCCCAGAG CAAAAAGCGA CTCCACATAG
6901 AAGACTACTC TGTTTCTCAG ACAACACTTG ACCAAGTATT TGTGAACTTT GCCAAGGACC
6961 AAAGTGATGA TGACCACTTA AAAGACCTCT CATTACACAA AAACCAGACA GTAGTGGACG
7021 TTGCAGTTCT CACATCTTTT CTACAGGATG AGAAAGTGAA AGAAAGCTAT GTATGAAGAA
7081 TCCTGTTCAT ACGGGGTGGC TGAAAGTAAA GAGGAACTAG ACTTTCCTTT GCACCATGTG
7141 AAGTGTTGTG GAGAAAAGAG CCAGAAGTTG ATGTGGGAAG AAGTAAACTG GATACTGTAC
7201 TGATACTATT CAATGCAATG CAATTCAATG CAATGAAAAC AAAATTCCAT TACAGGGGCA
7261 GTGCCTTTGT AGCCTATGTC TTGTATGGCT CTCAAGTGAA AGACTTGAAT TTAGTTTTTT
7321 ACCTATACCT ATGTGAAACT CTATTATGGA ACCCAATGGA CATATGGGTT TGAACTCACA
7381 CTTTTTTTTT TTTTTTGTTC CTGTGTATTC TCATTGGGGT TGCAACAATA ATTCATCAAG
7441 TAATCATGGC CAGCGATTAT TGATCAAAAT CAAAAGGTAA TGCACATCCT CATTCACTAA
7501 GCCATGCCAT GCCCAGGAGA CTGGTTTCCC GGTGACACAT CCATTGCTGG CAATGAGTGT
7561 GCCAGAGTTA TTAGTGCCAA GTTTTTCAGA AAGTTTGAAG CACCATGGTG TGTCATGCTC
7621 ACTTTTGTGA AAGCTGCTCT GCTCAGAGTC TATCAACATT GAATATCAGT TGACAGAATG
7681 GTGCCATGCG TGGCTAACAT CCTGCTTTGA TTCCCTCTGA TAAGCTGTTC TGGTGGCAGT
7741 AACATGCAAC AAAAATGTGG GTGTCTCTAG GCACGGGAAA CTTGGTTCCA TTGTTATATT
7801 GTCCTATGCT TCGAGCCATG GGTCTACAGG GTCATCCTTA TGAGACTCTT AAATATACTT
7861 AGATCCTGGT AAGAGGCAAA GAATCAACAG CCAAACTGCT GGGGCTGCAA GCTGCTGAAG
7921 CCAGGGCATG GGATTAAAGA GATTGTGCGT TCAAACCTAG GGAAGCCTGT GCCCATTTGT
7981 CCTGACTGTC TGCTAACATG GTACACTGCA TCTCAAGATG TTTATCTGAC ACAAGTGTAT
8041 TATTTCTGGC TTTTTGAATT AATCTAGAAA ATGAAAAGAT GGAGTTGTAT TTTGACAAAA
8101 ATGTTTGTAC TTTTTAATGT TATTTGGAAT TTTAAGTTCT ATCAGTGACT TCTGAATCCT
8161 TAGAATGGCC TCTTTGTAGA ACCCTGTGGT ATAGAGGAGT ATGGCCACTG CCCCACTATT
8221 TTTATTTTCT TATGTAAGTT TGCATATCAG TCATGACTAG TGCCTAGAAA GCAATGTGAT
8281 GGTCAGGATC TCATGACATT ATATTTGAGT TTCTTTCAGA TCATTTAGGA TACTCTTAAT
8341 CTCACTTCAT CAATCAAATA TTTTTTGAGT GTATGCTGTA GCTGAAAGAG TATGTACGTA
8401 CGTATAAGAC TAGAGAGATA TTAAGTCTCA GTACACTTCC TGTGCCATGT TATTCAGCTC
8461 ACTGGTTTAC AAATATAGGT TGTCTTGTGG TTGTAGGAGC CCACTGTAAC AATATTGGGC
8521 AGCCTTTTTT TTTTTTTTTT AATTGCAACA ATGCAAAAGC CAAGAAAGTA TAAGGGTCAC
8581 AAGTTTAAAC AATGAATTCT TCAACAGGGA AAACAGCTAG CTTGAAAACT TGCTGAAAAA
8641 CACAACTTGT GTTTATGGCA TTTAGTACCT TCAAATAATT GGCTTTGCAG ATATTGGATA
8701 CCCCATTAAA TCTGACAGTC TCAAATTTTT CATCTCTTCA ATCACTAGTC AAGAAAAATA
8761 TAAAAACAAC AAATACTTCC ATATGGAGCA TTTTTCAGAG TTTTCTAACC CAGTCTTATT
8821 TTTCTAGTCA GTAAACATTT GTAAAAATAC TGTTTCACTA ATACTTACTG TTAACTGTCT
8881 TGAGAGAAAA GAAAAATATG AGAGAACTAT TGTTTGGGGA AGTTCAAGTG ATCTTTCAAT
8941 ATCATTACTA ACTTCTTCCA CTTTTTCCAA AATTTGAATA TTAACGCTAA AGGTGTAAGA
9001 CTTCAGATTT CAAATTAATC TTTCTATATT TTTTAAATTT ACAGAATATT ATATAACCCA
9061 CTGCTGAAAA AGAAAAAAAT GATTGTTTTA GAAGTTAAAG TCAATATTGA TTTTAAATAT
9121 AAGTAATGAA GGCATATTTC CAATAACTAG TGATATGGCA TCGTTGCAAT TTACAGTATC
9181 TTCAAAAATA CAGAATTTAT AGAATAATTT CTCCTCATTT AATATTTTTC AAAATCAAAG
9241 TTATGGTTTC CTCATTTTAC TAAAATCGTA TTCTAATTCT TCATTATAGT AAATCTATGA
9301 GCAACTCCTT ACTTCGGTTC CTCTGATTTC AAGGCCATAT TTTAAAAAAT CAAAAGGCAC
9361 TGTGAACTAT TTTGAAGAAA ACACGACATT TTAATACAGA TTGAAAGGAC CTCTTCTGAA
9421 GCTAGAAACA ATCTATAGTT ATACATCTTC ATTAATACTG TGTTACCTTT TAAAATAGTA
9481 ATTTTTTACA TTTTCCTGTG TAAACCTAAT TGTGGTAGAA ATTTTTACCA ACTCTATACT
9541 CAATCAAGCA AAATTTCTGT ATATTCCCTG TGGAATGTAC CTATGTGAGT TTCAGAAATT
9601 CTCAAAATAC GTGTTCAAAA ATTTCTGCTT TTGCATCTTT GGGACACCTC AGAAAACTTA
9661 TTAACAACTG TGAATATGAG AAATACAGAA GAAAATAATA AGCCCTCTAT ACATAAATGC
9721 CCAGCACAAT TCATTGTTAA AAAACAACCA AACCTCACAC TACTGTATTT CATTATCTGT
9781 ACTGAAAGCA AATGCTTTGT GACTATTAAA TGTTGCACAT CATTCATTCA CTGTATAGTA
9841 ATCATTGACT AAAGCCATTT GCTGTGTTTT CTTCTTGTGG NTGNATATAT CAGGTAAAAT
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 9901 ATTTTCCAAA GAGCCATGTG TCATGTAATA CTGAACCCTT TGATATTGAG ACATTAATTT
 9961 GGACCCTTGG TATTATCTAC TAGAATAATG TAATACTGNA GAAATATTGC TCTAATTCTT
10021 TCAAAATGGT GCATCCCCCT TAAAANGTTC TATTTCCATA AGGATTTAGC TTGCTTATCC
10081 CTTCTTATAC CCTAAGATGA AGCTGTTTTT GTGCTCTTTG TTCATCATTG GCCCTCATTC
10141 CAAGCACTTT ACGCTGTCTG TAATGGGATC TATTTTTGCA CTGGAATATC TGAGAATTGC
10201 AAAACTAGAC AAAAGTTTCA CAACAGATTT CTAAGTTAAA TCATTTTCAT TAAAAGGAAA
10261 AAAGAAAAAA AATTTTGTAT GTCAATAACT TTATATGAAG TATTAAAATG CATATTTCTA
10321 TGTTGTAATA TAATGAGTCA CAAAATAAAG CTGTGACAGT TCTGTTAAAA AAAAAAAAAA
10381 AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
10441 AA
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From Page No.___

Important Results of BLAST SEARCH ABCR versus ABC1 (our clone)

I noticed that the homology between Our ABC1 sequence and ABCR started at position 338 on our sequence. But the putative ATG start (of ABC1) is at 503! ABCR is very close to ABC1 by homology search, and is 2273 residues long, but ABC1, as given in Chimini's paper in 2021 only.

So I translated rest of 5' region of our clone.

→ There is a possible 60 residue additional NH terminus on ABC1. It is preceded by an in frame stop codon! suggesting a true translation start site. Plus it looks a bit like a signal peptide!! Looks like anyone else failed to notice this — predicted consensus cleavage! Exactly the same residues are present in mouse ABC1, though one can back further (no info?)

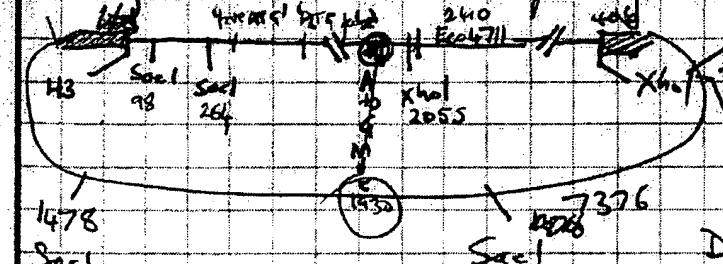
So we must ① redo all our constructs to include this

② Pray enough of the protein sequence to find real amino terminus.

NEW PRIMERS FOR 3' END OF HABC1

— may not need these. At first glance pCEP clone has 2 SAC1 sites in 5' end: and Xho1 site

If no Xho1 in pCEM32 can do this as a directional clone in 2kb band



No Xho1 in pCEM32 ✓

Digest with SSt1 and Xho1 in RE2:
8.25, 5.87, 3.0, 1.79, 1.11, 0.16

Isolate 1.79 Kb

Digest pCEMhABC1 with SSt1 and Xho1 — 1.67 and ~8 Kb

Isolate 8Kb

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Witnessed & Understood by me, | Date | Invented by | Date

Recorded by

The human ABC1 protein contains 60 more amino acids at its amino terminus than reported by other groups

The published protein sequence of human ABC1 protein, based on translated cDNA sequence, claims that the protein begins with the sequence MPSAGTLPWVQGII....(Langmann et al., Biochim. Biophys. Res. Comm. **257**, 29-33, 1999; GenBank Accession # AJ012376) and that the protein contains 2201 amino acids. This has been cited and assumed to be correct in all publications about ABC1 to date of which we are aware. This is important because authors have claimed to possess "full length cDNA clones", to design PCR primers to reproduce the full length coding region, to compare the full length coding region in normal individuals and those with diseases of HDL metabolism such as Tangier disease and familial hypo-alpha-lipoproteinemia, and to design expression plasmids to produce recombinant full length ABC1 protein based on this proposition. We believe it to be incorrect for the following reasons:
Our cDNA sequence of human ABC1 predicts an open reading frame that continues from the "Langmann" predicted start methionine, upstream for 60 more amino acids to a methionine codon, predicting that the true amino terminus of human ABC1 is:"
MACWPQLRLLLWKNLTFRRRQTCQLLLEVAWPLFIFLILISVRLSYPPYEQHECH
FPNKA....followed by the previously reported sequence beginning
MPSAGTLPWVQGII...There is an in-frame stop codon 6 to 9 nucleotides upstream from this location, so this would be the first methionine codon which could produce a continuous open reading frame. Alignment of our ABC1 cDNA sequence with GenBank sequences predicts a highly significant degree of similarity with other related ABC sequences, including the ABCR and ABC-C (also known as ABC3) transporters, which are also open reading frames for these 60 amino acids, implying that all three of these homologous proteins begin with sequences related to the amino terminal extension we propose for human ABC1. Presumably, the error in Langmann arose from their reliance on the published cDNA sequence of mouse ABC1 (Luciani et al. Genomics **21** 150-159, 1994). There, without direct evidence, they predicted an open reading frame of 2201 amino acids "potentially covering the whole coding region". In fact, they may have been in error in this presumption, because in their cDNA sequence (GenBank Accession

number X75926) there was an uncalled nucleotide "n" in this region, not allowing them to be sure that an open reading frame could have extended further upstream to an additional methionine codon. Following this potentially false lead, Langmann et al. designed PCR primers with which to obtain their human cDNA that did not extend far enough upstream to contain what we now propose to be the true extent of the coding region. In fact, if this undetermined "n" nucleotide in the mouse sequence is ignored, the mouse and human sequences of the "extra" 60 amino terminal amino acids are identical. In light of these results, it is highly likely that the true full length human ABC1 protein contains 2261 amino acids, and not the 2201 amino acids claimed by Langmann et al and in the subsequent publications to date which compare its sequence in normal individuals to those with Tangier disease. Recombinant DNA constructs useful to produce functional ABC1 protein or for gene therapy should contain the entire coding region for 2261 amino acids.

Direct proof of the true amino terminus of ABC1 can be obtained by amino terminal protein sequencing, followed by comparison to the sequence predicted by translation of the cDNA sequence. This can be accomplished by preparing membrane-bound protein preparations of macrophage or fibroblast cells, preferably after induction of ABC1 gene expression by cholesterol loading or 8-Br-cAMP treatment, as we have shown, to increase the yield of the desired protein. Partial purification can be performed by immunoprecipitation with anti-ABC1 antibodies, followed by SDS gel electrophoresis. The stained band corresponding to the molecular weight of 240 kDa is then excised after transfer of proteins from the gel to membrane, and amino terminal sequence determined by standard Edmon degradation procedures.

CVTHABC1 PROTEIN 2261 AA

Base pairs 291 to 7076 of CVThABC1 Nucleotide translated.

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   1 MACWPQLRLL LWKNLTFRRR QTCQLLLEVA WPLFIFLILI SVRLSYPPYE QHECHFPNKA
  61 MPSAGTLPWV QGIICNANNP CFRYPTPGEA PGVVGNFNKS IVARLFSDAR RLLLYSQKDT
 121 SMKDMRKVLR TLQQIKKSSS NLKLQDFLVD NETFSGFLYH NLSLPKSTVD KMLRADVILH
 181 KVFLQGYQLH LTSLCNGSKS EEMIQLGDQE VSELCGLPKE KLAAAERVLR SNMDILKPIL
 241 RTLNSTSPFP SKELAEATKT LLHSLGTLAQ ELFSMRSWSD MRQEVMFLTN VNSSSSSTQI
 301 YQAVSRIVCG HPEGGGLKIK SLNWYEDNNY KALFGGNGTE EDAETFYDNS TTPYCNDLMK
 361 NLESSPLSRI IWKALKPLLV GKILYTPDTP ATRQVMAEVN KTFQELAVFH DLEGMWEELS
 421 PKIWTFMENS QEMDLVRMLL DSRDNDHFWE QQLDGLDWTA QDIVAFLAKH PEDVQSSNGS
 481 VYTWREAFNE TNQAIRTISR FMECVNLNKL EPIATEVWLI NKSMELLDER KFWAGIVFTG
 541 ITPGSIELPH HVKYKIRMDI DNVERTNKIK DGYWDPGPRA DPFEDMRYVW GGFAYLQDVV
 601 EQAIIRVLTG TEKKTGVYMQ QMPYPCYVDD IFLRVMSRSM PLFMTLAWIY SVAVIIKGIV
 661 YEKEARLKET MRIMGLDNSI LWFSWFISSL IPLLVSAGLL VVILKLGNLL PYSDPSVVFV
 721 FLSVFAVVTI LQCFLISTLF SRANLAAACG GIIYFTLYLP YVLCVAWQDY VGFTLKIFAS
 781 LLSPVAFGFG CEYFALFEEQ GIGVQWDNLF ESPVEEDGFN LTTSISMMLF DTFLYGVMTW
 841 YIEAVFPGQY GIPRPWYFPC TKSYWFGEES DEKSHPGSNQ KRMSEICMEE EPTHLKLGVS
 901 IQNLVKVYRD GMKVAVDGLA LNFYEGQITS FLGHNGAGKT TTMSILTGLF PPTSGTAYIL
 961 GKDIRSEMST IRQNLGVCPQ HNVLFDMLTV EEHIWFYARL KGLSEKHVKA EMEQMALDVG
1021 LPSSKLKSKT SQLSGGMQRK LSVALAFVGG SKVVILDEPT AGVDPYSRRG IWELLLKYRQ
1081 GRTIILSTHH MDEADVLGDR IAIISHGKLC CVGSSLFLKN QLGTGYYLTL VKKDVESSLS
1141 SCRNSSSTVS YLKKEDSVSQ SSSDAGLGSD HESDTLTIDV SAISNLIRKH VSEARLVEDI
1201 GHELTYVLPY EAAKEGAFVE LFHEIDDRLS DLGISSYGIS ETTLEEIFLK VAEESGVDAE
1261 TSDGTLPARR NRRAFGDKQS CLRPFTEDDA ADPNDSDIDP ESRETDLLSG MDGKGSYQVK
1321 GWKLTQQQFV ALLWKRLLIA RRSRKGFFAQ IVLPAVFVCI ALVFSLIVPP FGKYPSLELQ
1381 PWMYNEQYTF VSNDAPEDTG TLELLNALTK DPGFGTRCME GNPIPDTPCQ AGEEEWTTAP
1441 VPQTIMDLFQ NGNWTMQNPS PACQCSSDKI KKMLPVCPPG AGGLPPPQRK QNTADILQDL
1501 TGRNISDYLV KTYVQIIAKS LKNKIWVNEF RYGGFSLGVS NTQALPPSQE VNDAIKQMKK
1561 HLKLAKDSSA DRFLNSLGRF MTGLDTRNNV KVWFNNKGWH AISSFLNVIN NAILRANLQK
1621 GENPSHYGIT AFNHPLNLTK QQLSEVALMT TSVDVLVSIC VIFAMSFVPA SFVVFLIQER
1681 VSKAKHLQFI SGVKPVIYWL SNFVWDMCNY VVPATLVIII FICFQQKSYV SSTNLPVLAL
1741 LLLLYGWSIT PLMYPASFVF KIPSTAYVVL TSVNLFIGIN GSVATFVLEL FTDNKLNNIN
1801 DILKSVFLIF PHFCLGRGLI DMVKNQAMAD ALERFGENRF VSPLSWDLVG RNLFAMAVEG
1861 VVFFLITVLI QYRFFIRPRP VNAKLSPLND EDEDVRRERQ RILDGGGQND ILEIKELTKI
1921 YRRKRKPAVD RICVGIPPGE CFGLLGVNGA GKSSTFKMLT GDTTVTRGDA FLNKNSILSN
1981 IHEVHQNMGY CPQFDAITEL LTGREHVEFF ALLRGVPEKE VGKVGEWAIR KLGLVKYGEK
2041 YAGNYSGGNK RKLSTAMALI GGPPVVFLDE PTTGMDPKAR RFLWNCALSV VKEGRSVVLT
2101 SHSMEECEAL CTRMAIMVNG RFRCLGSVQH LKNRFGDGYT IVVRIAGSNP DLKPVQDFFG
2161 LAFPGSVLKE KHRNMLQYQL PSSLSSLARI FSILSQSKKR LHIEDYSVSQ TTLDQVFVNF
2221 AKDQSDDDHL KDLSLHKNQT VVDVAVLTSF LQDEKVKESY V*
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